Date syrup derived polyphenols attenuate angiogenic responses and exhibits anti-inflammatory activity mediated by VEGF and COX-2 expression in endothelial cells

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11 **ABSTRACT**

12 Bioactive components such as polyphenols, present in many plants, are purported to
13 have anti-inflammatory and anti-angiogenic properties. Date syrup, produced from
14 date fruit of the date palm tree has traditionally been used to treat a wide range of
15 diseases with etiologies involving angiogenesis and inflammation. It was
16 hypothesized that polyphenols in date syrup reduce angiogenic responses such as
17 cell migration, tube formation and matrix metalloproteinase activity in an
18 inflammatory model by exhibiting anti-inflammatory activity mediated by vascular
19 endothelial growth factor (VEGF) and the prostaglandin enzyme cyclooxygenase-2
20 (COX-2) in endothelial cells. Date syrup polyphenols at 60μg/mL and 600μg/mL
21 reduced inflammation and suppressed several stages of angiogenesis, including
22 endothelial cell migration, invasion, matrix metalloproteinase activity and tube
23 formation without evidence of cytotoxicity. VEGF and COX-2 expression induced by
24 tumor necrosis factor - alpha (TNF- α) at both gene expression and protein level,
25 was significantly reduced by date syrup polyphenols in comparison to untreated
26 cells. In conclusion, polyphenols in date syrup attenuated angiogenic responses and
27 exhibited anti-inflammatory activity mediated by VEGF and COX-2 expression in
28 endothelial cells.

29

30 **Keywords:** angiogenesis, polyphenols, cyclooxygenase-2, vascular endothelial
31 growth factor, inflammation.

32 **Abbreviations**

33

34 ACE; Advanced Chromatography Technologies LTD

35 ANOVA; Analysis of Variance

36 BME; Basement Membrane Extract

37 COX-2; Cyclooxygenase-2

38 DMEM; Dulbecco's modified Eagle's medium

39 ECACC; European Collection of Animal cell cultures

40 ELISA; Enzyme Linked Immunosorbant Assay

41 GAPDH; Glyceraldehyde-3-Phosphate Dehydrogenase

42 GUSB; Beta-glucuronidase

43 HECV; Human Vascular Endothelial Cell

44 HPLC; High Performance Liquid Chromatography

45 IL-1β; Interleukin-1beta

46 IL-6; Interleukin-6

47 IL-8; Interleukin-8

48 IL-17β; Interleukin-17beta

49 LC; Liquid chromatography

50 LC-ESI MS; Liquid chromatography – electrospray ionization mass spectrometry

51 LC/MSD; Liquid chromatography/mass spectrometer detector

52 MMP-2; Matrixmetalloproteinase-2

53 MMP-9; Matrixmetalloproteinase-9

54 MT1-MMP; Membrane associated Type 1- Matrix Metalloproteinase

55 PBS; Phosphate Buffered Saline

56 PCR; Polymerase Chain Reaction

57 PPDS; extracted Date Syrup Polyphenol

58 Prostaglandin E2

59 TGF-β1; Tumor Growth Factor-beta1

60 TNF-α; Tumor Necrosis Factor- alpha

61 TIMP; Tissue Inhibitor of Metalloproteinase

62 Δct; Comparative Cycle Threshold method

63 VEGF; Vascular Endothelial Growth Factor

64 **1. Introduction**

65

66 Angiogenesis encompasses the formation of new blood vessels from pre-existing
67 vasculature and is a tightly regulated and coordinated process involving both pro-
68 and anti-angiogenic factors [1]. The development of new capillaries from pre-existing
69 micro-vessels is a critical event in wound repair and tissue regeneration [2] and is a
70 time controlled physiological process [3]. When the homeostatic balance between
71 stimulation and inhibition is shifted, excessive angiogenesis ensues resulting in
72 inflammatory associated angiogenesis. Inflammatory associated angiogenesis is
73 commonly associated with oxidative stress [4]. Inflammation is a paramount process
74 in defense against pathogenic invasion and it can induce adverse effects on tissue
75 over time [5].

76

77 Key regulators involved in the angiogenic process include gelatinase matrix
78 metalloproteinases (MMPs), which degrade the extracellular matrix of endothelial
79 cells. MMP-2 and MMP-9 have been associated with inflammation and are key
80 factors involved in inflammatory associated angiogenesis [6]. Another important
81 physiological and pathological mediator is the cyclooxygenase (COX) enzyme COX-
82 2. The COX-2 enzyme catalyzes prostanoid synthesis and is involved in the
83 arachidonic acid pathway associated with prostaglandin E2 (PGE2) production [7].
84 COX-2 up regulates vascular endothelial growth factor (VEGF) another important
85 factor promoting vascular development and therefore promoting inflammatory
86 associated angiogenesis [8].

87

88 Natural compounds have traditionally been used to prevent and treat various
89 illnesses worldwide. As a result, there has been a growing interest in assessing the
90 role of plant and food-based bioactive compounds such as polyphenols with reported
91 antioxidant [9,10], anti-inflammatory [11] and antimicrobial [12] activity. Anti-
92 angiogenic properties in endothelial cells have been described for several
93 polyphenol compounds including quercetin [13], epigallocatechin gallate [14],
94 curcumin [15] and resveratrol [16].
95
96 Date fruits, and date fruit products including date syrup from different cultivars, have
97 traditionally been used as alternative medicine in the treatment of a range of
98 ailments including stomach and intestinal disorders, fever, edema, bronchitis and in
99 wound repair [17]. Several bioactive compounds such as polyphenols have been
100 identified within date syrup, which suggest a possible rationale for date syrup's
101 perceived traditional medicinal application. Date syrup has been found to have a
102 high content of polyphenol compounds such as flavonoids, tannins, carotenoids and
103 anthocyanins [18]. Given the increasing number of literature focusing on the role of
104 bioactive compounds such as polyphenols as anti-angiogenic and anti-inflammatory
105 agents, the action of polyphenols in date syrup, in relation to endothelial cells
106 angiogenic and inflammatory responses have not been investigated.
107 Hence it was hypothesized that polyphenols derived from date syrup reduce
108 angiogenic responses in an inflammatory model of endothelial cells and this
109 reduction is mediated by reduced expression of VEGF and COX-2.
110 To address this hypothesis, the objective of this study was to determine the pre-
111 treating of date syrup polyphenols (60µg/mL and 600µg/mL) on angiogenic
112 responses associated with tube formation, cell migration, cell invasion and MMP

113 activity in human endothelial cells. Furthermore, the pro-inflammatory cytokine levels
114 secreted by endothelial cells stimulated with TNF-alpha and pre-treated with date
115 syrup polyphenols was determined. Additionally, the study further determined
116 whether changes in angiogenic and inflammatory responses in endothelial cells
117 treated with date syrup polyphenols reduced the expressions of COX-2 and VEGF.

118 **2. Methods and materials**

119

120 **2.1. Chemicals and reagents**

121

122 XAD-2 Resin, Folin-Ciocalteau reagent and all polyphenol standards including Gallic
123 acid were obtained from Sigma (Sigma Aldrich, United Kingdom). HPLC grade
124 methanol and formic acid was obtained from Fisher Scientific (UK). TRIzol® was
125 obtained from Life Technologies.

126

127 **2.2. Extraction and chemical analysis of date syrup polyphenol**

128

129 Date syrup was produced from the date fruit cultivar Khadrawi, belonging to the
130 family *Arecaceae*, genus *Phoenix* and species *dactylifera* during the wet seasons of
131 2012-2013. The date syrup was raw and unprocessed; it was stored at 4 °C on
132 receipt. Date syrup phenolic fraction was extracted according to the method
133 described by [19]. Unprocessed date syrup (50 g) was mixed with 250 mL of
134 acidified water (pH2) for 24 hours at room temperature; the mixture was filtered
135 through cotton wool to remove un-dissolved solid particles. XAD-2 resin (Supelco)
136 (approximately 47 g) was initially conditioned in 2M HCl for 1 hour, and further
137 conditioned by soaking in 1:1 methanol and water for pre-swelling overnight. The
138 slurry was packed into a glass column (MBL) and the solution removed to give an
139 approximate bed volume of 1 x 50 cm^3 and rinsed with 1 L of deionized water. The
140 date syrup solution was passed slowly at 1mL / minute through the packed resin
141 column, followed by 250 mL of acidified water (pH2) and deionized water (300 mL)
142 (ELGA LabWater). Polyphenol fractions were finally eluted with 300 mL pure

143 methanol. A 50 mL of collected methanol extract was concentrated to dryness under
144 vacuum at 40 °C. The extract (PPDS) was stored at -80 °C, subjected to chemical
145 analysis and dissolved accordingly for cell culture treatment in cell medium. The
146 quantification of total phenolic content of date syrup was determined by the Folin-
147 Ciocalteu colorimetric assay based on the procedure previously identified by Al-Farsi
148 and colleagues [20]. Gallic acid was used as a spectrophotometric standard (0-100
149 mg/mL) and results were expressed and means ± SD mg of gallic acid equivalents
150 (GAE) per 100 g of date syrup. Measurements were taken in triplicate.
151 Extracted date syrup polyphenols were analyzed using HPLC based on the method
152 by [21]. Chromatographic analysis was carried out with an Agilent 1200 LC (Agilent,
153 Berkshire, UK). Data was processed with Agilent ChemStation software. An ACE
154 C18-300 column (250 x 7.75 mm) was used for the separation of phenolic
155 compounds at 30 °C and the mobile phase consisted (A) water and formic acid
156 (95:5) and (B) methanol which was previously degassed in a sonication system twice
157 for 50 minutes at 25 °C.
158 The solvent gradient system consisted of 35% B for 20 minutes, 45% B for 35
159 minutes, 80% B for 5 minutes and 95% B for 5 minutes followed by a post-time
160 isocratic run of 35% B for 30 minutes between injections. The flow rate was 0.8
161 mL/minute and the injection volume was maintained at 20 µl. The monitoring
162 wavelengths were 280 and 320 nm respectively. Subsequent quantification and
163 identification of polyphenols was assessed using an LC-ESI MS system consisted of
164 an Agilent LC 1200 series (Agilent, Berkshire, UK) coupled to a LC/MSD Trap XCT
165 Ultra (Agilent) mass spectrometer equipped with an ESI source and ion trap mass
166 analyzer. The drying gas temperature was set at 300 °C with a rate of 3.0 L/minute,
167 a spray voltage of 3.2 kV and the samples were scanned at *m/z* values of 120-1000

168 amu using positive electrospray ionization. Data was processed with Agilent
169 ChemStation software and identified phenolic compounds were quantified by
170 correlating the measured peak area with the calibration curve and *m/z* obtained with
171 reference compounds.
172
173 **2.2. Experimental design**
174
175 **2.2.1 Cell culture and treatments**
176
177 Human HECV endothelial cell line was purchased from the European Collection of
178 Animal Cell Cultures (ECACC) (Salisbury, UK). HECV cells were grown in
179 Dulbecco's modified Eagle's medium (DMEM) (Gibco) (high glucose) supplemented
180 with 2 mM L-glutamine, 100 IU/mL penicillin and 200 µg/mL streptomycin (Gibco,
181 Life Technologies) and 10% v/v fetal calf serum (LabTech International). Cells were
182 maintained at 37 °C under a 5% CO_2 and 95% air atmosphere at constant humidity
183 (New Brunswick Galaxy 170R, Eppendorf). HECV cells were used within the
184 passage range of 7 -15 for consecutive experiments in this study.
185
186 **2.2.2. Cell viability assessment using CellTiter® Blue**
187
188 HECV cells were seeded into sterile 96 well round bottomed polystyrene microtitre
189 plates (Corning Costar Ltd, NY, USA) and treated with extracted date syrup
190 polyphenols (PPDS) dissolved and rehydrated in culture medium at a maximum
191 concentration of 1200 µg/mL Extracted date syrup polyphenols were diluted over a
192 concentration range (60 - 1200 µg/mL) to a final concentration of 1200 µg/mL and

193 added to HECV cells. Following a 24-hour incubation, the supernatant was
194 aspirated, HECV cells were washed twice with phosphate buffered saline (PBS)
195 (Gibco) and the CellTiter® Blue (Promega) assay was performed as described by Lo
196 and colleagues [22].
197
198 **2.2.3. Determination of VEGF, IL-8 and IL-6 levels in culture supernatants**
199
200 HECV endothelial cells were seeded into 6-well plates and allowed to reach 80%
201 confluence. Cells were treated with and without TNF- α (20 ng/mL) one hour prior to
202 the addition of extracted PPDS at concentrations of 60 µg/mL and 600 µg/mL.
203 Culture supernatants were collected at 6 and 24 hour and stored at -80 °C. VEGF,
204 IL-8 and IL-6 were measured using enzyme-linked immunosorbant assays (ELISA; R
205 & D Systems, Abingdon, UK) using 96-well microtitre plates in accordance with the
206 manufacturer's instructions [23].
207
208 **2.2.4. *In vitro* cell invasion assay**
209
210 The effect of extracted PPDS on HECV cell invasion was assessed using a tissue
211 culture transwell insert, with an 8-µm pore size polyethylene terephthalate
212 membrane and a 96-well companion plate included in the *in vitro* Angiogenesis
213 Assay Endothelial Cell Invasion kit (Cultrex Trevigen Inc., Gaithersburg, MD, USA),
214 all reagents were included within the kit. The invasion assay was performed as
215 described previously [24]. Briefly, on day 1, 50 µl (0.5 X) of Basement Membrane
216 Extract (BME) coating solution was placed in each well of the top invasion chamber.
217 HECV cells were starved overnight in serum free DMEM. The following day (day 2)

HECV cells were harvested and seeded into the top chamber at 4 x 10^5 cells per well in 50 µl of serum-free media. Extracted PPDS prepared at 60 µg/mL and 600 µg/mL in serum-free media was added at 150 µl to each well of the bottom invasion chamber in the presence and absence of TNF- α (20 ng/mL) and incubated at 37 °C containing 5% CO_2. After 16 hours incubation medium in both chambers was aspirated and each well washed with washing buffer (1X). This was followed by 150 µl of cell dissociation / Calcein-AM solution added to each well and incubated at 37 °C for 1 hour. The top chamber was removed and the bottom plate measured using a fluorescence plate reader (Tecan infinite M200, Männedorf, Switzerland) at 485 nm excitation and 520 nm emissions. Experiments were performed in triplicate and results expressed as percentage of cell invasion relative to control.

2.2.5. *In Vitro* migration assay

Cell migration was evaluated using the migration assay according to the method described [25]. HECV cells at a cell density of 1 X 10^6 / mL were seeded into 6-well plates and incubated to form a confluent monolayer. The supernatant was removed and replaced with 60 µg/mL and or 600 µg/mL extracted PPDS with and without the chemo-attractants TNF- α (20 ng/mL) and VEGF (15 ng/mL). The monolayer was then scratched with a P200 pipette tip across the well to generate a scratch approximately 0.5 mm wide. Cell migration was calculated and expressed as % migration as an expression of the control.

2.2.6. *In Vitro* tube formation determination

243 To study tubular formation in HECVs, the *in vitro* Angiogenesis Assay Tube
244 Formation Kit (Cultrex, Trevigen Inc., Gaithersburg, MD, USA) was used according
245 to the manufacturer's instructions and with slight modification according to [26].
246 Briefly, all reagents were included within the kit. Initially, growth factor-reduced
247 Basement Membrane Extract (BME) was aliquot into a 96-well plate and incubated
248 for 16 hours at 37 °C. Subsequently, HECV cells were incubated with a fluorescent
249 Calcein-AM solution (2 μM) for 30 minutes. HECV cells (1 x 10^5 per well) were
250 seeded in supplemented DMEM containing 60 μg/mL and or 600 μg/mL extracted
251 PPDS with and without TNF- α (20 ng/mL) and VEGF (15 ng/mL). Following a 10-
252 hour incubation period, three random fields were selected per well and tube-like
253 structures were viewed, monitored and photographed with a camera attached to a
254 fluorescence microscope (Axiovert 25 (20 x), Zeiss). Each determination was
255 performed in triplicate.
256
257 **2.2.7. Detection of MMP-related proteins in supernatants of HECV cells using**
258 **ELISA-based arrays**
259
260 To test for MMP-related proteins in supernatants of treated HECV cells with
261 extracted PPDS, an ELISA-based method using Quantibody chips from RayBiotech
262 (Norcross, GA, USA) was used according to the manufacturer's instruction [27],
263 allowing the simultaneous measurement of a range of MMP's with specific emphasis
264 on MMP-2. HECVs were seeded into 6-well plates and allowed to reach 80%
265 confluence. Subsequently, HECVs were washed and treated with and without TNF-
266 α (20 ng/mL) in the presence of extracted PPDS at concentrations of 60 μg/mL and
267 or 600 μg/mL, and incubated for 24 hours at 37 °C. Whereby the supernatant was

268 collected and assayed. According to the manufacturers instructions the arrays were
269 blocked with blocking solution for 1 hour at room temperature, 100 μl of sample
270 supernatant was added to each well and the array incubated at 4 °C for 16 hours.
271 Samples were decanted and the wells washed 5 times with PBS, followed by the
272 detection antibody cocktail added to each well and the plates incubated for 16 hours
273 at 4 °C. The detection antibody was decanted and washed 5 times with PBS, the
274 supplied fluorescent dye labeled-streptavidin was added and the array incubated for
275 a further 16 hours at 4 °C, washed 5 times with PBS, and the fluorescent signal
276 measured on a InnoScan 710 scanner (Innopsys, Chicago, Illinois, USA).
277

278 **2.2.8. Determination of gene expression using Real-Time PCR**

279

280 HECV cells were seeded into 6-well plates and treated with and without TNF- α (20
281 ng/mL) in the presence of extracted PPDS at concentrations of 60 μg/mL and or 600
282 μg/mL, and incubated for 24 hours at 37 °C. Total RNA was extracted and isolated
283 using TRIzol® according to the manufacturer's protocol. RNA was eluted in RNAse-
284 free water (ELGA LabWater) and stored at -80 °C. RNA concentration and purity
285 were assessed using the Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher
286 Scientific, Wilmington, USA) system. Samples with an $A_{260/280}$ ratio of RNA purity of
287 1.7 – 2.0 were subsequently used for cDNA conversion. cDNA conversion was
288 achieved using a High Capacity cDNA Reverse Transcription kit (Applied
289 Biosystems) according to the manufacturer's instruction. Changes in the expression
290 of *VEGF* and *COX-2* gene expression were assessed by one-step quantitative PCR
291 [28] (Taqman System, Applied Biosystems, Madrid, Spain). Amplification was
292 performed using 100 ng/mL cDNA for a total reaction of 20 μl in micro-well plates

293 (Thermo Fisher Scientific) covered by optical adhesive covers, and using Taqman
294 Universal Master mix. Primers and probes were the following; *VEGF-A* (spanning
295 exon 3 / exon 4; ID; Hs00900055_m1), *COX-2* (spanning exon 5 / exon 6; ID;
296 Hs00153133_m1), *GAPDH* (spanning exon 8 / exon 9; ID; Hs03929097_g1) and
297 *GUSB* (spanning exon 8 / exon 9; ID; Hs00929627_m1). The quantitative PCR used
298 the Applied Biosystems 7500 Real-Time PCR System under the following conditions;
299 incubation at 50 °C for 2 minutes and 95 °C for 10 minutes, samples were amplified
300 for 40 cycles at 95 °C for 15 seconds, followed by 60 °C for 1 minute. The
301 expression level of target genes was normalized to the endogenous controls *GAPDH*
302 and *GUSB* using the ΔC_T method for quantification [29]. All assays were performed
303 in triplicate.
304
305 **2.3. Statistical analyses**
306
307 All experiments were carried out three times independently and in triplicates and did
308 not involve a power analysis. All data are presented as means ± SD. Significant
309 differences were determined by one-way analysis of variance with Bonferroni *post-*
310 *hoc* analysis used for multiple comparisons within different HECV endothelial cell
311 treatment and different PPDS concentrations. The level of significance was defined
312 as p <0.05 as statistically significant using superscript labeling. Statistical analysis
313 was performed using GraphPad Prism® Version 6 software (GraphPad Software, Inc.
314 La Jolla, CA, USA).

315 **3. Results**

316

317 **3.1.The effect of extracted date syrup polyphenols on HECV viability and cell**
318 **invasion**

319

320 The quantification and determination of date syrup polyphenols is outlined in Fig. 1
321 and Table 1. The nine main peaks identified using ESI-MS methodology allowed the
322 identification of date syrup polyphenols based on their characteristic molecular ions
323 and its comparison against the Folin-Ciocalteau assay. The most common
324 polyphenol compounds identified in date syrup are the cinnamic acids and their
325 derivatives, namely *p*-Coumaric acid, 3-Caffeoylquinic acid, and Caffeic acid.
326 HECV cell viability expressed as % was analyzed by the CellTiter® Blue assay and is
327 outlined in Fig. 2. HECV viability upon treatment with a range of extracted PPDS
328 concentrations (60 - 1200 µg/mL) showed no statistical difference (p >0.05) in any of
329 the concentrations indicating the extracted PPDS concentrations had no cytotoxic
330 effect on HECV cells. Since no significant changes in cell viability were observed
331 against tested compounds, two concentrations of extracted PPDS namely 600
332 µg/mL and 60 µg/mL were used throughout the remainder of the study.

333

334 Cell motility and extracellular matrix invasion are fundamental processes within
335 angiogenesis; therefore the effect of date syrup polyphenols on invasion capacity
336 using a double chamber was investigated. Fig. 3 illustrates the effect of the addition
337 of extracted PPDS on the inflammatory stimulant TNF-α and the angiogenic growth
338 factor VEGF. Extracted PPDS at 60 µg/mL demonstrated the most significant
339 invasive capacity following incubation with VEGF and / or TNF-α (Fig. 3b and 3c)

with the addition of TNF-α (10% increase) the greatest in comparison to VEGF ($p < 0.05$).

3.2. Extracted PPDS inhibit angiogenic responses in endothelial cells

For blood vessel formation, endothelial cells are required to differentiate and reorganize to enable assembly of vascular like capillary structures. Endothelial cells such as HECV are able to form highly branched capillary-like structures when cultured on BME obtained from mouse sarcoma cells. The effect of extracted PPDS on angiogenesis in affecting *de novo* the formation of *in vitro* capillary-like structures was examined; as shown in Fig. 4a and 4b respectively; incubation and pre-treatment of HECV cultures on BME coated plates with extracted PPDS at concentrations of 60 μg/mL and 600 μg/mL reduced the stimulated tube-like differentiation in comparison to TNF-α and VEGF treated HECV cells. This resulted in unconnected structures exhibiting no edges (Fig. 4a and 4b). Tube formation was monitored and branching revealed (Fig. 4c) that branch points significantly decline in a dose dependent manner in TNF-α and VEGF stimulated endothelial cells ($p < 0.01$), this further demonstrates a significant interaction between treatment groups and concentration of PPDS, whereby the greater concentration of PPDS had the greatest significance. This finding suggests that date syrup polyphenols exhibit anti-angiogenic effect in a dose-dependent manner.

Similarly, the migration assay measuring endothelial migration (Fig. 5) demonstrated that 600 μg/mL extracted PPDS decreased TNF-α migration of endothelial cells by greater than 50% (Fig. 5) in comparison to the control, 60 μg/mL of PPDS also

365 decreased percentage migration but the highest PPDS concentration, was more
366 effective in reducing migration as compared to HECV cells alone. Furthermore, this
367 effect was also observed for TNF-α and VEGF stimulated migration response, with
368 extracted PPDS responding and significantly decreasing locomotion ($p < 0.05$) in
369 VEGF greater than TNF-α. This indicates a clear *in vitro* anti-angiogenic response
370 for date syrup polyphenols.
371
372 **3.3. Polyphenols in date syrup inhibit MMP-2 activity in endothelial cells**
373
374 To assess and confirm the role of MMP-2 in promoting *in vitro* angiogenesis in
375 endothelial cells and their response to extracted PPDS, microarray analysis was
376 performed to assess MMP and tissue inhibitors of matrix metalloproteinases (TIMPs)
377 activity in endothelial cells. HECVs were stimulated with TNF-α, treated with
378 extracted PPDS and assessed for MMP-2 activity. In TNF-α induced HECV (Fig. 6),
379 microarray analysis revealed that TNF-α treated HECV, MMP-2 secreted in culture
380 media at 24 hours was significantly increased (Fig. 6). In comparison, HECV pre-
381 treatment with extracted PPDS significantly decreased the gelatinolytic activity of
382 MMP-2. This interaction effect was also observed in a concentration dependent
383 manner (Fig. 6), with a higher concentration of extracted PPDS (600 μg/mL) more
384 effective at reducing MMP-2 activity. MMP-2 activity is regulated by TIMPs; therefore
385 TIMP-1 and TIMP-2 release in culture media was measured, but no significant
386 change (p >0.05) in TIMP activity by extracted PPDS was found (data not shown).
387 Therefore the MMP-2/TIMP ratio decreased as a result of MMP-2 production. This
388 finding indicates that angiogenesis inhibition by polyphenols involves MMP-2
389 production in endothelial cells.

390

391 **3.4. Assessment of VEGF, IL-8 and IL-6 release in endothelial cells**

392

393 Extracted PPDS concentrations of 60 µg/mL and 600 µg/mL significantly decreased
394 in a time and dose dependent manner the basal levels of VEGF and the increases in
395 VEGF induced by TNF-α (Fig. 7ai and 7aii). It is noteworthy that while both
396 concentrations of extracted PPDS decrease VEGF levels, 60 µg/mL achieved a
397 decline of VEGF in a time dependent manner by significantly reducing VEGF over 24
398 hours; statistical analysis demonstrated this effect was significant ($p < 0.05$) for both
399 time and treatment. Endothelial cells such as HECV secrete VEGF, IL-8 and IL-6 as
400 regulators of inflammation and angiogenesis in culture supernatants over time.
401 Under basal conditions, HECV's secreted VEGF, IL-8 and IL-6 (Fig. 7), however the
402 levels of IL-6 were the lowest (ranging from 7-40 pg/mL per 10^6 cells after 24 hours)
403 (Fig. 7c). This is in contrast to the levels of VEGF (from 40-300 pg/mL after 24 hours)
404 and IL-8 (ranging from 100-600 pg/mL) (Fig. 7b). The pro-inflammatory cytokine
405 TNF-α up regulated both VEGF and IL-8 activity (treatment effect) in endothelial
406 cells. It was observed that the addition of extracted PPDS decreased VEGF, IL-8
407 and IL-6 in a simultaneous time and concentration dependent manner with statistical
408 significance across all cytokine activity ($p < 0.05$) (Fig. 7a, 7b and 7c). The addition
409 of extracted PPDS also reduced the expression of both VEGF and IL-8 in HECV
410 supernatants after TNF-α stimulation. Under basal conditions, VEGF and IL-8 were
411 down regulated by extracted PPDS, whereas very little difference was observed for
412 IL-6. These observations suggest that extracted PPDS modulate simultaneous anti-
413 angiogenic (decreasing VEGF) and anti-inflammatory (decreasing IL-8) responses in
414 endothelial cells associated with angiogenesis and inflammation.

415

416 **3.5. Extracted date syrup polyphenols inhibit TNF-α stimulated VEGF and COX-
417 2 activity and expression**
418
419 The activation of COX-2 and VEGF is a tightly associated and regulated pathway in
420 the angiogenic and inflammatory response cascade. This investigation examined
421 extracted PPDS effects directly on COX-2 and VEGF gene expression by real time
422 PCR analysis. COX-2 gene expression was induced by the pro-inflammatory
423 cytokine TNF-α, but extracted PPDS caused a significant reduction in TNF-α induced
424 COX-2 gene expression (Fig. 8a) demonstrating both a concentration and treatment
425 effect ($p < 0.05$). This effect was also observed for HECV treated with extracted
426 PPDS alone when compared to the COX-2 gene expression of HECV (control). This
427 supports an anti-inflammatory response observed in Fig. 7 for VEGF, IL-8 and IL-6.
428 Interestingly, VEGF gene expression was also reduced in HECV cells induced with
429 TNF-α and treated with 600 μg/mL extracted PPDS (Fig. 8b) in comparison to HECV
430 cells induced with TNF-α.

431 **4. Discussion**

432

433 The anti-angiogenic and anti-inflammatory effect of bioactive compounds found
434 commonly in foods and their role in the prevention and treatment of inflammatory
435 and angiogenic-associated pathogenesis has been previously reported [30].

436

437 This investigation demonstrated the effect of quantified date syrup polyphenols on
438 angiogenic and inflammatory responses in human HECV endothelial cells. Moreover,
439 the study demonstrates that extracted PPDS inhibited COX-2 and VEGF gene
440 expression in TNF-α induced HECV, furthermore PPDS down regulated pro-
441 angiogenic growth factor VEGF, pro-inflammatory cytokine IL-8 and matrix
442 metalloproteinase activity MMP-2, which are direct responses, associated with
443 angiogenesis and inflammation. Additionally, date syrup polyphenol treatment of
444 TNF-α HECV cells inhibited cell proliferation, migration, invasion capacity and *in vitro*
445 capillary tube formation. These findings demonstrate the importance of endothelial
446 cells in an inflammatory model and demonstrate the anti-inflammatory effect of
447 polyphenols in date syrup and highlight the anti-angiogenic properties of extracted
448 PPDS on human HECV endothelial cell function.

449

450 Date syrup polyphenols consist predominantly of cinnamic acids such as *p*-
451 Coumaric acid, cinnamic acid, gallic acid, ferrulic acid, chlorogenic acid, catechin
452 derivatives and hydrocaffeic acid. Date syrup is also rich in flavanols, flavonoids,
453 tannins and carotenoids [18]. Most of the identified polyphenols and antioxidant-
454 associated compounds have demonstrated bioactive behavior and anti-inflammatory
455 and cancer progression delay routinely associated with angiogenesis [31,32,33].

456 This study supports current literature examining different polyphenols in inflammation
457 and angiogenesis, therefore suggesting that polyphenols in date syrup contribute to
458 the anti-angiogenic and anti-inflammatory results observed.
459
460 The inflammatory process involves the accumulation of immune cells and the
461 subsequent release of pro-inflammatory cytokines and chemokines [34]. The
462 addition of TNF-α to endothelial cells creates an 'inflammatory environment' that
463 contributes to inflammatory associated disorders such as Crohn's disease and retinal
464 disorders [35]. The activation of endothelial cells by TNF-α leads to an increase in
465 COX-2 expression, which stimulates increased expression and release of VEGF [8].
466 To investigate the anti-angiogenic and anti-inflammatory responses of extracted
467 PPDS, this study created an inflammatory environment in endothelial cells to model
468 inflammation and angiogenic responses. Stimulation with TNF-α increased the
469 expression of cytokines IL-8 and IL-6, COX-2 and VEGF, which are secreted in
470 response to an inflammatory environment. Extracted PPDS significantly attenuated
471 the cytokine-mediated inflammatory response (IL-8) indicating the potential of
472 extracted PPDS in preventing inflammation in endothelial cells. Data from this study,
473 supports previous research demonstrating that polyphenols regulate COX-2 and
474 inflammatory cytokine secretion [36, 37, 38].
475
476 Endothelial cell angiogenesis has developed an adjunct mechanism contributing to
477 inflammation [4]. Increased tissue vasculature enhances the tissue inflammatory
478 responses of recruitment and activation associated with endothelial cells [3]. This
479 study assessed the effect of extracted PPDS on angiogenic properties of HECV
480 cells. The stimulation of HECV with TNF-α or VEGF increased angiogenic responses

481 of proliferation, migration, invasion and tube formation. Extracted PPDS inhibited
482 TNF-α and VEGF induced migration, invasion capacity and tube formation, which
483 indicate that polyphenols in date syrup in combination have anti-angiogenic activity.
484
485 Furthermore, the anti-angiogenic response of extracted PPDS was strongly
486 associated with significant reduction of COX-2 and VEGF gene expression, which
487 confirms our hypothesis that the inhibition of angiogenic responses of polyphenols in
488 date syrup in endothelial cells involves a decrease in COX-2 and VEGF gene
489 expression. The COX-2 pathway has been implicated in the concept of the
490 'angiogenic switch' [34] whereby heightened COX-2 expression in a given tissue
491 environment initiates a shift towards pro-angiogenesis by stimulating tissue formation
492 [38]. Stimulation of COX-2 consequently induces the activation and production of
493 VEGF [9]. Down regulation of COX-2 and VEGF pathways have been associated
494 with anti-angiogenic, anti-inflammatory and anti-carcinogenic activity of polyphenols
495 and polyphenol rich foods [38, 39, 40, 41] in *in vitro* and *in vivo* models of
496 angiogenesis and inflammation. This study has demonstrated and observed the anti-
497 angiogenic responses of extracted PPDS in an inflammatory endothelial function and
498 has enhanced the knowledge and understanding of the potential pathway of the
499 combination of polyphenols in date syrup in anti-inflammatory and anti-angiogenic
500 prevention.
501
502 Various stimulants initiate or enhance gelatinase expression and inflammatory
503 cytokine stimulation in diverse cell types. Examples include TNF-α, transforming
504 growth factor β1 (TGF- β1), and interleukin 1β (IL-1β). The activation and release of
505 specific cytokines, chemokines, and growth factors at tissue sites under stress

506 activates nearby endothelial cells towards the defective site, a process that
507 progresses via up-regulation of MMP activity. MMPs are a family of zinc-dependent
508 endopeptidases, which are involved in the breakdown of the extracellular matrix
509 (ECM) during tissue remodeling [42]. Excessive breakdown of ECM is associated
510 with pathological inflammatory diseases such as arthritis, autoimmune skin diseases
511 and tumor invasion [43]. Among MMPs, MMP-2 and MMP-9 (gelatinase A and B) are
512 implicated in angiogenesis and inflammation [44].
513

514 MMP-2 is secreted from cells as latent zymogens, which are maintained by their
515 specific endogenous inhibitors, the tissue inhibitor of metalloproteinase (TIMP)–2
516 and TIMP-1. Once activated, MMP-2 is able to digest components of the basement
517 membrane such as type IV collagen and fibronectin [45]. This suggests that
518 gelatinizes such as MMP-2 are likely to promote the progression of angiogenesis
519 and inflammation by inducing a collagen poor environment, which facilitates cell
520 migration, proliferation and vascular formation. Date syrup polyphenols inhibit MMP-
521 2 activity by maintaining a collagen rich environment thus preventing angiogenesis
522 associated inflammation.

523

524 There are several potential limitations to this study, there is limited evidence on the
525 use of the HECV cell line in the assessment of angiogenesis and inflammation,
526 VEGF was the only direct marker at both protein and gene levels assessing
527 angiogenesis and the extent of MMP-2 and MMP-9 relationship and activity needs
528 further evaluation since only MMP-2 was investigated. However the inhibition of
529 MMP-9 points to all endothelial cells being subjected to inflammation and

530 angiogenesis once activated and previous research have utilized the HECV
531 endothelial cell line looking at IL-17B [46], serine protease matriptase-2 associated
532 with cancer progression [47] and endothelial cell function linked with adhesion and
533 migration [48]. Further research is needed to effectively assess the role of HECV
534 endothelial cells in angiogenesis

535 This study reports the significant effects of date syrup polyphenols on stimulated
536 endothelial inflammation and angiogenesis in assessing angiogenic and
537 inflammatory responses in human HECV endothelial cells. This investigation
538 demonstrated extracted PPDS significantly inhibited TNF-α mediated endothelial
539 invasion, migration, MMP-2 activity and tube formation. In addition, the anti-
540 inflammatory effect of extracted PPDS in HECV was confirmed by an observed
541 reduction of pro-inflammatory cytokines IL-8, IL-6 and the growth factor VEGF. The
542 reduction in COX-2 and VEGF gene expression would support the view, that date
543 syrup polyphenol's anti-angiogenic and anti-inflammatory activity occurs through
544 COX-2 and VEGF regulatory pathways.

545

546 **Acknowledgments**

547

548 The authors would like to thank Marina Ross, Andrew Thomas, Stephen Potter and
549 Richard Rowlands for their assistance and support throughout the study.
550 There is no external funding associated with this study. The authors declare that
551 there is no conflict of interest associated with this manuscript.

REFERENCES

[1] Bouïs D, Kusumanto Y, Meijer C, Mulder NH, & Hospers GA. A review on pro- and anti-angiogenic factors as targets of clinical intervention. Pharmacological Research 2006;53:89-103.

[2] Varinska L, van Wijhe M, Belleri M, Mitola S, Perjesi P, Presta M, et al J. Anti-angiogenic activity of the flavonoid precursor 4-hydroxychalcone. European Journal of Pharmacology 2012;691:125-133.

[3] Folkman J. Angiogenesis: an organizing principle for drug discovery?. Nature Reviews Drug Discovery 2007;6:273-286.

[4] Soares R. Angiogenesis in the metabolic syndrome. In Oxidative Stress, Inflammation and Angiogenesis in the Metabolic Syndrome. Netherlands; Springer: 2009:85-99.

[5] Imhof BA, & Aurrand-Lions M. Angiogenesis and inflammation face off. Nature Medicine 2006;12:171-172.

[6] Kessenbrock K, Plaks V, & Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010;141:52-67.

[7] Wu G, Luo J, Rana JS, Laham R, Sellke FW, & Li J. Involvement of COX-2 in VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial cells. Cardiovascular Research 2006;69:512-519.

577

578 [8] Angelo LS, & Kurzrock R. Vascular endothelial growth factor and its
579 relationship to inflammatory mediators. Clinical Cancer Research
580 2007;13:2825-2830.
581

582 [9] Costa C, Incio J, & Soares R. Angiogenesis and chronic inflammation: cause or
583 consequence?. Angiogenesis 2007;10:149-166.
584

585 [10] Martín-Sánchez AM, Cherif S, Ben-Abda J, Barber-Vallés X, Pérez-Álvarez, JÁ,
586 Sayas-Barberá E. Phytochemicals in date co-products and their antioxidant
587 activity. Food Chemistry 2014;158:513-520.
588

589 [11] Kassim M, Achoui M, Mustafa MR, Mohd MA, & Yusoff KM. Ellagic acid,
590 phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro
591 anti-inflammatory activity. Nutrition Research 2010;30:650-659.
592

593 [12] Liu X, Li J, Wang Y, Li T, Zhao J, Zhang C. Green tea polyphenols function as
594 prooxidants to inhibit Pseudomonas aeruginosa and induce the expression of
595 oxidative stress-related genes. Folia Microbiologica 2013;58:211-217.
596

597 [13] Igura K, Ohta T, Kuroda Y, & Kaji K. Resveratrol and quercetin inhibit
598 angiogenesis in vitro. Cancer Letters 2001;171:11-16.
599

600 [14] Trompezinski S, Denis A, Schmitt D, & Viac J. Comparative effects of
601 polyphenols from green tea (EGCG) and soybean (genistein) on VEGF and IL-

602 8 release from normal human keratinocytes stimulated with the
603 proinflammatory cytokine TNFα. Archives of Dermatological Research
604 2003;295:112-116.
605
606 [15] Maheshwari RK, Singh AK, Gaddipati J, & Srimal RC. Multiple biological
607 activities of curcumin: a short review. Life Sciences 2006;78:2081-2087.
608
609 [16] Cao Y, Fu ZD, Wang F, Liu HY, & Han R. Anti-angiogenic activity of
610 resveratrol, a natural compound from medicinal plants. Journal of Asian Natural
611 Products Research 2005;7:205-213.
612
613 [17] Tahraoui, A, El-Hilaly J, Israili ZH, Lyoussi B**.** Ethnopharmacological survey of
614 plants used in the traditional treatment of hypertension and diabetes in south-
615 eastern Morocco (Errachidia province). Journal of Ethnopharmacology
616 2007;110:105-117.
617
618 [18] Dhaouadi K, Raboudi F, Estevan C, Barrajón E, Vilanova E, Hamdaoui M, &
619 Fattouch S. Cell viability effects and antioxidant and antimicrobial activities of
620 Tunisian date syrup (Rub El Tamer) polyphenolic extracts. Journal of
621 Agricultural and Food Chemistry 2011;59:402-406.
622
623 [19] Yao L, Datta N, Tomás-Barberán FA, Ferreres F, Martos I, Singanusong R.
624 Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand
625 Leptospermum honeys. Food Chemistry 2003;81;159-68.
626

627 [20] Al-Farsi M, Alasalvar C, Morris A, Baron M, Shahidi F.Comparison of
628 antioxidant activity, anthocyanins, carotenoids, and phenolics of three native
629 fresh and sun-dried date (Phoenix dactylifera L.) varieties grown in Oman.
630 Journal of Agricultural and Food Chemistry 2005:53;7592-7599.
631
632 [21] Weston RJ, Mitchell KR, Allen KL. Antibacterial phenolic components of New
633 Zealand manuka honey. Food chemistry 1999;64:295-301.
634
635 [22] Lo HW, Cao X, Zhu H, & Ali-Osman F. Constitutively activated STAT3
636 frequently coexpresses with epidermal growth factor receptor in high-grade
637 gliomas and targeting STAT3 sensitizes them to Iressa and alkylators. Clinical
638 Cancer Research 2008;14:6042-6054.
639
640 [23] Kim IB, Kim DY, Lee SJ, Sun MJ, Lee MS, Li H et al.. Inhibition of IL-8
641 production by green tea polyphenols in human nasal fibroblasts and a549
642 epithelial cells. Biological and Pharmaceutical Bulletin 2006;29:1120-5.
643
644 [24] Liu X, Yu J, Jiang LU, Wang A, Shi F, Ye H et al.. MicroRNA-222 regulates cell
645 invasion by targeting matrix metalloproteinase 1 (MMP1) and manganese
646 superoxide dismutase 2 (SOD2) in tongue squamous cell carcinoma cell lines.
647 Cancer Genomics-Proteomics 2009;6:131-139.
648
649 [25] Nepal M, Choi HJ, Choi BY, Kim SL, Ryu JH, Kim DH et al.. Anti-angiogenic
650 and anti-tumor activity of Bavachinin by targeting hypoxia-inducible factor-1α.

651 European Journal of Pharmacology 2012; 691:28-37.

652

653 [26] Kim Y, Kim BH, Lee H, Jeon B, Lee YS, Kwon MJ, Kim TY. Regulation of skin
654 inflammation and angiogenesis by EC-SOD via HIF-1α and NF-κB pathways.
655 Free Radical Biology and Medicine 2011;51:1985-95.

656

657 [27] Yang CC, Lin CY, Wang HS, Lyu SR. Matrix metalloproteases and tissue
658 inhibitors of metalloproteinases in medial plica and pannus-like tissue
659 contribute to knee osteoarthritis progression Plos 2013:e79662.

660

661 [28] von Rahden BH, Stein HJ, Pühringer F, Koch I, Langer R, Piontek G et al..
662 Coexpression of cyclooxygenases (COX-1, COX-2) and vascular endothelial
663 growth factors (VEGF-A, VEGF-C) in esophageal adenocarcinoma. Cancer
664 research 2005;65:5038-44.

665

666 [29] Livak KJ, and Schmittgen TD. Analysis of relative gene expression data using
667 real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 2001;25:402-
668 408.

669

670 [30] Fan TP, Yeh JC, Leung KW, Yue PY, & Wong RN: Angiogenesis: from plants
671 to blood vessels. Trends in Pharmacological Sciences 2006; 27:297-309.

672

673 [31] Tang FY, & Meydani M. Green tea catechins and vitamin E inhibit angiogenesis
674 of human microvascular endothelial cells through suppression of IL-8
675 production. Nutrition and Cancer 2001;41:119-125.

676

677 [32] Rodriguez SK, Guo W, Liu L, Band MA, Paulson EK, & Meydani M. Green tea
678 catechin, epigallocatechin- 3- gallate, inhibits vascular endothelial growth
679 factor angiogenic signaling by disrupting the formation of a receptor complex.
680 International Journal of Cancer 2006; 118:1635-1644.
681
682 [33] Jung JE, Kim HS, Lee CS, Park DH, Kim, YN, Lee MJ et al.. Caffeic acid and
683 its synthetic derivative CADPE suppress tumor angiogenesis by blocking
684 STAT3-mediated VEGF expression in human renal carcinoma cells.
685 Carcinogenesis 2007;28:1780-1787.
686
687 [34] Hanahan D, & Folkman J. Patterns and emerging mechanisms of the
688 angiogenic switch during tumorigenesis. Cell 1996;86:353-364.
689
690 [35] Hatoum OA, & Binion DG. The vasculature and inflammatory bowel disease.
691 Contribution to pathogenesis and clinical pathology. Inflammatory Bowel
692 Diseases 2005;11:304-313.
693
694 [36] Murakami A, & Ohigashi H. Targeting NOX, INOS and COX- 2 in inflammatory
695 cells: Chemoprevention using food phytochemicals. International Journal of
696 Cancer 2007;121:2357-2363.
697
698 [37] Li A, Dubey S, Varney ML, Dave BJ, & Singh RK. IL-8 directly enhanced
699 endothelial cell survival, proliferation, and matrix metalloproteinases production
700 and regulated angiogenesis. The Journal of Immunology 2003;170:3369-3376.
701

702 [38] Scoditti E, Calabriso N, Massaro M, Pellegrino M, Storelli C, Martines G et al.. Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a potentially protective mechanism in atherosclerotic vascular disease and cancer. Archives of Biochemistry and Biophysics 2012.527:81-89.
703
704
705
706
707

708 [39] Corona G, Deiana M, Incani A, Vauzour D, Dessì MA, & Spencer JP. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. Biochemical and Biophysical Research Communications 2007;362:606-611.
709
710
711
712

713 [40] Bedran TBL, Spolidorio DP, & Grenier D. Green tea polyphenol epigallocatechin-3-gallate and cranberry proanthocyanidins act in synergy with cathelicidin (LL-37) to reduce the LPS-induced inflammatory response in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. Archives of Oral Biology 2015; 60:845-853.
714
715
716
717
718

719 [41] Medda R, Lyros O, Schmidt JL, Jovanovic N, Nie L, Link BJ et al. Anti inflammatory and anti angiogenic effect of black raspberry extract on human esophageal and intestinal microvascular endothelial cells. Microvascular Research 2015;97:167-180.
720
721
722
723

724 [42] Gialeli C, Theocharis AD, & Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FeBS Journal 2011;278:16-27.
725
726

727

728 [43] Yang CC, Lin CY, Wang HS, & Lyu SR. Matrix metalloproteases and tissue
729 inhibitors of metalloproteinases in medial plica and pannus-like tissue
730 contribute to knee osteoarthritis progression. Plos 2013;e79662.
731

732 [44] Nagase H, Visse R, & Murphy G. Structure and function of matrix
733 metalloproteinases and TIMPs. Cardiovascular Research 2006;69:562-573.
734

735 [45] Annabi B, Lachambre MP, Bousquet-Gagnon N, Pagé M, Gingras D, &
736 Béliveau R. Green tea polyphenol (−)-epigallocatechin 3-gallate inhibits MMP-2
737 secretion and MT1-MMP-driven migration in glioblastoma cells. Biochimica et
738 Biophysica Acta (BBA)-Molecular Cell Research 2002; 1542:209-220.
739

740 [46] Sanders AJ, Guo X, Mason MD, & Jiang WG. IL-17B can impact on endothelial
741 cellular traits linked to tumour angiogenesis. Journal of Oncology: Open Access
742 ID: 817375 2010.
743

744 [47] Webb SL, Sanders AJ, Mason MD, & Jiang WG. Matriptase-2 inhibits HECV
745 motility and tubule formation in vitro and tumour angiogenesis in vivo.
746 Molecular and Cellular Biochemistry 2013;375:207-217.
747

748 [48] Yin Y, Sanders AJ, Feng L, & Jiang WG. Knockdown of AAMP Impacts on
749 HECV Cell Functions In Vitro and Affects the Expression of VE-Cadherin.
750 Angiology: Open Access 2014.
751

List of captions

Table 1.

Table 1. Quantification and determination of total and individual phenolic compounds in date syrup.

Figure 1.

Fig. 1. **Chemical composition of extracted date syrup polyphenols.** HPLC chromatogram representing extracted date syrup polyphenols at 280 nm. Fractions a-i were quantitatively identified in triplicates on three separate occasions (n=9) using LC-MS and the resulting peaks represent the following polyphenols (left to right): (a) Gallic acid; (b) hydrocaffeic compound; (c) catechin derivative; (d) Cinnamic acid derivative; (e) 3-Caffeoylquinic acid; (f) Caffeic acid; (g) *p*-Coumaric acid; (h) Unknown compound; (i) 3-O-caffeoylshikimic acid

Figure 2.

Fig. 2. **Effect of extracted date syrup polyphenols on HECV viability.** Cell viability after incubation for 24 hours with 60 - 1200 µg/mL extracted date syrup polyphenol (PPDS) by the CellTiter® Blue assay. Cell viability is presented as percentage means ± SD of control (n=3). Data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. No significant difference ($p > 0.05$) was observed compared with untreated cells (X).

Figure 3.

Fig. 3. Effect of extracted PPDS on HECV invasion capacity. Cells incubated for 24 hour with 60 µg/mL and 600 µg/mL extracted date syrup polyphenols (PPDS) and

assessed using the *in vitro* Angiogenesis Assay Endothelial Cell Invasion kit; a) effect of HECV cells with 60 µg/mL and 600 µg/mL PPDS on invasion capacity for 24 hours, b) effect of pre-treated HECV with 60 µg/mL and 600 µg/mL extracted PPDS for 24 hours with stimulant VEGF c) effect of pre-treated HECV cells with 60 µg/mL and 600 µg/mL extracted PPDS for 24 hours with stimulant TNF-α. Invasion capacity is presented as percentage invasion means ± SD of independent replicates (n=3). Data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction between different HECV endothelial cell treatment group and PPDS concentrations. Means without a common superscript are significantly different from each other ($p < 0.05$ *vs.* control).

Figure 4.

Fig. 4. Effect of extracted PPDS on HECV tube formation. Culture plates (96-well plate) were coated with growth factor reduced BME overnight. HECVs (1 x 10^5 cells) were grown overnight before being treated with 60 µg/mL & 600 µg/mL extracted date syrup polyphenol (PPDS) in the presence of a) TNF-α and b) VEGF for 10 hours. Images of tube formation were taken (x 20). Figures are representative of the whole culture treatment. c) Semi-quantitation of capillary-like formation of HECV. HECV cells stimulated with TNF-α and or VEGF and treated with extracted PPDS were monitored for tube formation. Data represents means tubule branch points per field ± SD (n = 6 fields) per treatment (n = 3). Data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. Means without a common superscript are significantly different from each other ($p < 0.05$) of treatments and concentrations.

Figure 5.

Fig. 5. Effect of extracted PPDS on cell migration. Cell migration was evaluated by the migration assay. HECV cells were stimulated with TNF-α and or VEGF, pre-treated with 60 μg/mL or 600 μg/mL extracted PPDS and scratched through the monolayer. Migration percentages relative to control (equated to 100%) were analyzed relative to microscope cells per field. Figures are representative of the whole culture treatment. Results are presented as means ± SD of replicates (n =4) and data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. Means without a common superscript are significantly different from each other (p <0.05) of cell treatments and different PPDS concentrations.

Figure 6.

Fig. 6. MMP-2 activity on HECV cells treated with extracted PPDS. HECV cells were stimulated with the pro inflammatory cytokine TNF-α and treated with 60 μg/mL and 600 μg/mL extracted date syrup polyphenol (PPDS) for 24 hours. MMP activity was assessed using a fluorescence microarray. Data is presented as means ± SD of independent replicates (n =3) and data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. Means without a common superscript are significantly different from each other (p <0.05) representative of independent PPDS concentrations and different treatment.

Figure 7.

Fig. 7. **Regulation of cytokine and VEGF activity by extracted PPDS**. HECV cells were stimulated with TNF-α and treated with 60 μg/mL and 600 μg/mL extracted date syrup polyphenol (PPDS) respectively. After 6 and 24 hour incubation, supernatants were collected and examined for a) VEGF, b) IL-8 and c) IL-6 activity

using ELISA methodology. Data is presented as means ± SD of independent replicates in triplicate (n =6) of different treatments at different PPDS concentrations. Data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. Means without a common superscript are significantly different from each other (p <0.05) of treatments and concentrations.

Figure 8.

Fig. 8. Effect of extracted PPDS on gene expression of COX-2 and VEGF. HECV cells stimulated with TNF-α and treated with 600 µg/mL of extracted date syrup polyphenol (PPDS) for 24 hours was investigated for relative gene expression of COX-2 and VEGF using quantitative real-time PCR to baseline levels of HECV cells (control). Quantitation was normalized against the endogenous controls *GAPDH* and *GUSB*. Results are means ± SD of triplicate replicates in triplicate (n =9). Data were analyzed using one-way ANOVA using Bonferroni *post hoc* correction. Means without a common superscript are significantly different from each other (p <0.05) representative of concentrations of PPDS and different treatments .