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1 Polyphenols extracted from red grape pomace by a
2 surfactant based method show enhanced collagenase and
3 elastase inhibitory activity
4

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15

16 **Short title:** Collagenase and elastase inhibitory activity of polyphenols separated by CGA

17 **Abstract**

18 **BACKGROUND:** The aim of this study is to separate polyphenols from grape pomace using a
19 surfactant-based separation, Colloidal Gas Aphrons (CGA) and to investigate their inhibitory
20 activity against skin relevant enzymes, collagenase and elastase. Ethanolic (EE) and hot water
21 crude extracts (HWE) were produced first and then the CGA generated using TWEEN20 were
22 applied resulting in polyphenols enriched fractions (CGA-EE and CGA-HWE, ethanol and hot
23 water extracts derived fractions respectively).
24

25 **RESULTS:** Both crude extracts inhibited the enzymes in a dose-dependent manner however,
26 further extraction by CGA led to fractions with higher inhibitory efficiency against collagenase.
27 Although gallic acid was the main component of the CGA-HWE, others such as kaempferol
28 must have contributed to its potency which was over six times more than gallic acid's. The

1 CGA-EE was found to be about four times more efficient than its crude extract and over six
2 times more efficient than gallic acid in collagenase's inhibition; quercetin was the major
3 polyphenol in this fraction.

4

5 **CONCLUSION:** It is evident that ethanol and hot water extraction processes led to different
6 polyphenols composition and thus different inhibitory activity against collagenase and elastase.
7 Further separation with CGA increased the inhibitory potency of both extracts against
8 collagenase. Overall the results here showed the potential application of the CGA fractions
9 from grape extracts in cosmetics.

10

11 **Keywords:** colloidal gas aphrons, grape pomace, polyphenols, collagenase, elastase

12 **Abbreviations:** AAAPVN, N-Succ-Ala-Ala-Ala-p-nitroanilide; AOAC; association of
13 analytical communities; CGA, colloidal gas aphrons; ChC; *C. hystoliticum* collagenase Type
14 IA; FALGPA; N-[3—(2-furyl)acryloyl]-Leu-Gly-Pro-Ala; ECM; extracellular matrix;
15 GAE_{280nm}, gallic acid equivalents based on total phenol index measure at 280nm; GAE_{760nm},
16 gallic acid equivalents based on Folin index measure at 760nm; PPE; porcine pancreatic
17 elastase.

18

19 **INTRODUCTION**

20 Over the past two decades, research on the use of natural products, particularly
21 polyphenols, in beauty products has been active but challenging (1). Polyphenols with a
22 hydroxyl group (-OH) attached to an aromatic benzene ring (C₆H₅-) naturally occur in plants
23 and are therefore abundant in our diet (eg: vegetables, fruits, nuts, seeds and flowers), and have
24 been extensively studied for their protective health effects against cardiovascular diseases and
25 cancers (2). Moreover, they have been proven to exhibit significant antioxidant activity, as well
26 as a UV protection effect which are very crucial for skin care products formulation (3).

1 Green tea is the most widely studied plant for its cosmetic applications. Green tea
2 polyphenols extract incorporated in derma gels were found to display significant antioxidant
3 activity and prevent adverse effects of UV radiation by improving the elasticity of the skin
4 (4,5). Catechins and epigallocatechin gallate from green tea and cocoa beans extracts were
5 found to possibly contribute to this effect (5–7). In addition, catechin could stabilise the
6 structure of collagen suggesting the involvement of hydrogen bonding and hydrophobic
7 interactions as major forces in its stabilisation (8). Moreover Sin & Kim (9) found that the
8 flavonols, particularly quercetin and kaempferol exhibited higher collagenase inhibitory
9 activity than flavones/isoflavones. In a recent study, Wittenauer et al. (10) found that free
10 phenolic acids, particularly gallic acid extracted from grape had the most potent inhibitory
11 activity against both collagenase and elastase. However, it is worth mentioning that the
12 inhibitory concentration of polyphenols varies between studies and samples (268 μ M –
13 1000 μ M); this is partly due to the variations in polyphenols composition. Also the size of
14 polyphenols restricts their permeation into the epidermal and corium layers (11) which could
15 hinder their application in cosmetics.

16 Surfactants are often used in cosmetics products to address the problem with the
17 permeation of the desired molecules. Surfactants in their micellar form can help in the
18 solubilisation of compounds (12) hence increasing its permeation through the skin and
19 promoting absorption by lowering the interfacial tension at the skin surface. The delivery of
20 resveratrol and curcumin has been improved by the presence of surfactants in pig skin (13),
21 and the acceleration of hydrocortisone and lidocaine has been observed on hairless mouse skin
22 by using TWEEN80 (14). Therefore, using a surfactant based extraction method is an
23 advantage as the product is extracted in a media (surfactant solution) that is suitable and
24 possibly optimum for its formulation, which can lead to process simplification.

1 In our group, we investigated a surfactant based extraction method, Colloidal Gas
2 Aphrons (CGA), for the separation of polyphenols from grape (15). CGA are microbubbles
3 (10-100 μ m) generated by intense stirring (>8000rpm) of a surfactant solution above its critical
4 micelle concentration. CGA are composed of an inner core gas surrounded by a thin layer film.
5 The type of surfactant used to generate CGA determines the charge of the outer surface of the
6 layer, which could be positive, negative or non-charged and oppositely or non-charged
7 molecules will adsorb resulting in their effective separation (16).

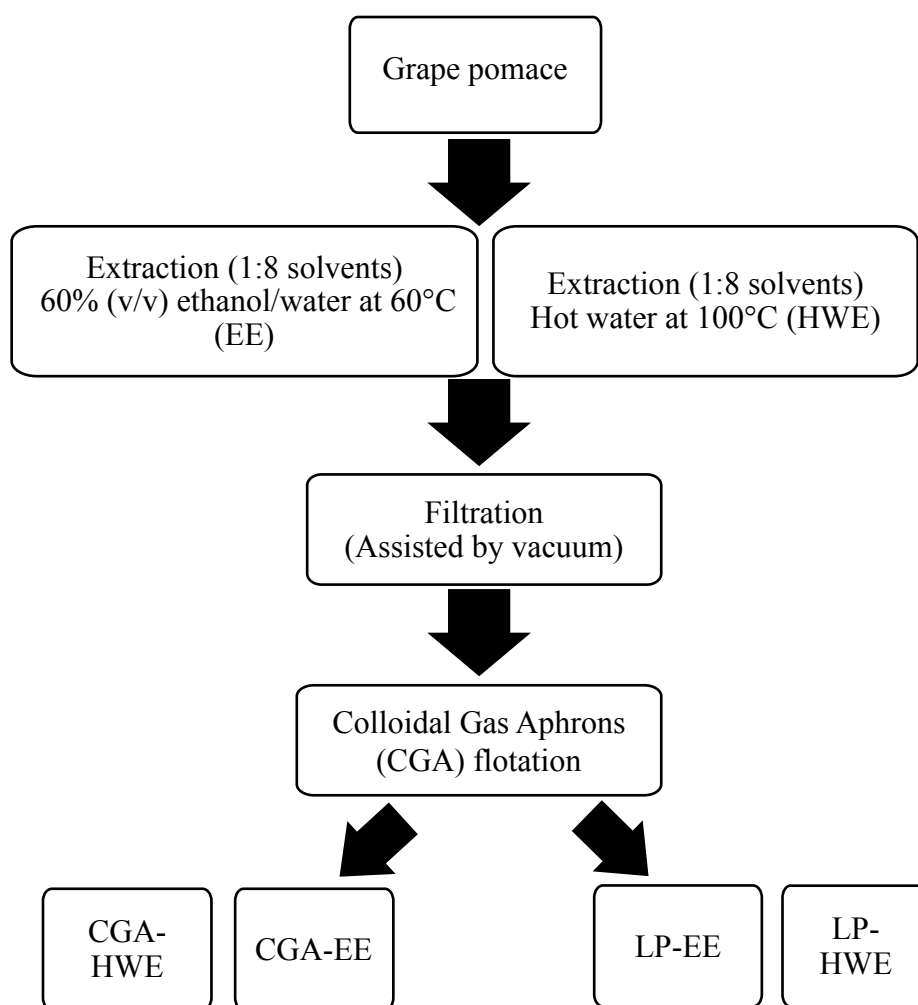
8 In the present study the aim was to determine if the extraction of polyphenols by the
9 CGA method led to enhanced *in vitro* inhibitory activity against *Clostridium histolyticum*
10 collagenase (ChC) and porcine pancreatic elastase (PPE) enzymes. The relationship between
11 polyphenol composition of the raw and the CGA extracts, and their inhibitory activity were
12 also investigated in order to identify the key polyphenols responsible for these activities. To
13 the best of our knowledge, this is the first study on the potential inhibitory activity of red grape
14 pomace extracts and their CGA fractions against ChC and PPE.

15

16 **MATERIALS AND METHODS**

17 Grape pomace (Barbera) provided by wineries in Northern Italy was oven dried at 60°C
18 until the residual moisture content was <5%, and milled into particle size of <2mm. The
19 phenolic extracts were obtained by ethanol-aqueous extraction using 60% (v/v) and hot water
20 extraction at 60°C and 100°C in a shaking water bath (100rpm) in circular motion for 2 hours
21 and 1 hour, respectively (See Figure 1 for full extraction process). For both extractions, the
22 ratio of solute to solvents used was 1:8 according to (17). The extracts were kept at -20°C
23 freezer until further use. Extractions were carried out in triplicate.

1 *C. hystoliticum* collagenase type IA (ChC), N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala
 2 (FALGPA), porcine pancreatic elastase (PPE) type III, N-Succ-Ala-Ala-p-nitroanilide
 3 (AAAPVN), phenol crystals and BCA reagents were obtained from Sigma (St. Louis, MO).
 4 The polyphenols standards used for HPLC analysis and inhibition studies were gallic acid
 5 ($\geq 95\%$), caffeic acid ($\geq 95\%$), epicatechin ($\geq 95\%$), p-coumaric acid ($\geq 95\%$), benzoic acid
 6 ($\geq 99.5\%$), *trans*-resveratrol ($\geq 95\%$), quercetin ($\geq 95\%$), malvidin-3-o-glucoside ($\geq 95\%$),
 7 cyanidin-3-o-glucoside ($\geq 95\%$), petunidin-3-o-glucoside ($\geq 95\%$) and delphinidin-3-o-
 8 glucoside ($\geq 95\%$) from Sigma (St. Louis, MO); and procyanidin (B2 $\geq 90\%$), from Fluka
 9 (Buchs, Switzerland). All solvents were of HPLC grade or LC-MS grade.



10

11 Figure 1: Schematic representation of the extraction of polyphenolic compounds present in grape
 12 pomace. The whole procedure was performed in triplicate (n = 3). EE, ethanol extract, HWE, hot water
 13 extract, CGA-EE; Aphron phase of EE, CGA-HWE; Aphron phase of HWE, LP-EE, liquid phase of
 14 EE and LP-HWE; liquid phase of HWE

1 **Characterisation of grape pomace**

2 **Phenolic compounds**

3 The grape pomace extracts, liquid and CGA fractions recovered were characterised for
4 its total phenolics and anthocyanins. **Total phenolics** were measured by:

5 (i) direct measurement based on the absorbance reading at 280nm. Results were expressed by
6 gallic acid equivalents (GAE_{280nm}) by means of calibration curve with standard gallic acid
7 ranging from 0-150mg/L (17). (ii) Folin-Ciocalteu method (18). The results were expressed as
8 gallic acid equivalents (GAE_{760nm}).

9 **Total anthocyanins** content was determined by applying the method from AOAC (19).
10 This method is based on the anthocyanins structural transformation that occurs with a change
11 in pH and commonly referred as pH differential method. The results were expressed as mg/L
12 malvidin-3-glucoside equivalents (ME).

13

14 **Non-phenolic compounds**

15 **Total proteins** were quantified according to the bicinchoninic acid assay (BCA) (20).
16 Briefly, 100µl of standard or sample was mixed with 2 mL of the BCA working reagent (copper
17 sulphate solution:BCA solution at a ratio of 1:50). The mixture was allowed to stand at 37°C
18 for 30 mins, and then allowed to cool at room temperature for 5 mins. Finally, the absorbance
19 for each sample/standard was read at 562nm within 8 mins with water as a blank. Bovine serum
20 albumin (0-1.0mg/L) was used as a standard for protein quantification.

21 **Total sugar** content was performed adopting the method from Dubois et al. (21). In test
22 tubes, 0.4mL of glucose standard/sample was added followed by 0.2mL of 5% phenol solution.
23 Subsequently, 1mL of sulphuric acid was pipetted direct to the solution and vortexed. The
24 mixture was allowed to stand for 20-30mins to cool off. The absorbance of the mixture was

1 read at 490nm and a calibration curve was constructed with different concentrations (10-
2 100mg/L) of glucose standard. The results were expressed as mg/L glucose equivalent.

3

4 **Determination of polyphenols composition by HPLC**

5 Separation of the polyphenols was performed using an Agilent HPLC 1100 series system
6 equipped with a degasser, a quaternary pump and a photodiode array detector (Agilent,
7 Waldbronn, Germany) with Chemstation software. The column used was a C18 HiChrom
8 column (150 mm x 4.6 mm i.d; 5µm particle size and 100 Å pore size; part no.EXL-121-
9 1546U) operated at 25°C.

10 The mobile phase consisted of 2% formic acid (v/v) and 5% acetonitrile (v/v) in water
11 (mobile phase A) and 2% formic acid (v/v) in acetonitrile (mobile phase B) using the following
12 gradient: 5-15% B (15 mins), 15-30% B (15 mins), 30-50% B (10 mins), 50-95% B (5 mins)
13 and 95-5% B (5 mins), at a flow rate of 1mL/min. The total run was 50 mins. The pre time of
14 10 mins was allowed for re-equilibrating. The injection volume was 20µL for pure standards
15 and 100µL for grape extracts. The polyphenols were monitored simultenesouly at 280nm
16 (hydroxybenzoic acids and flavanols), 320nm (hydroxycinnamic acids and stilbenes), 365nm
17 (flavonols) and 520nm (anthocyanins).

18

19 **Identification of polyphenols by LC-MS**

20 The LC-MS analyses were carried out using a Thermo Scientific Accela HPLC with PDA
21 UV/Vis detector interfaced to a Thermo Scientific LTQ Orbitrap XL with ESI source.
22 Chromatographic separation was carried out using an Ace-5 C18 column; 150 x 2.1 mm, 5µm
23 particle, 300 Å pore (part no. 221.1502). All samples were analysed without dilution and in 10
24 fold dilution. Dilutions were done in mobile phase A2 buffer (0.1% formic acid (v/v) in LC-

1 MS water). Mobile phase B2 buffer was 0.1% formic acid (v/v) in acetonitrile. Injections
2 volumes were 20 μ L. The following gradient was used: 0 min 5% B2; 5-15% B2 (15mins), 15-
3 30% B2 (15mins), 30-50% B2 (5mins), 50-95% B2 (5mins), 95-5% B2 (5mins) and 5% B2
4 (10mins), at a flow rate of 200 μ L/min.

5 The MS parameters were as follow: a standard of caffeic acid was infused into the MS
6 source alongside the HPLC flow at 20% mobile phase B; using a T-piece the source and
7 transmission settings were optimised for both positive and negative ion modes. The salient
8 settings were as follows: sheath gas flow at 45, aux gas at 10, sweep gas at 0 and the capillary
9 temperature was at 300°C. For the positive mode, the source voltage was 5Kv, capillary voltage
10 was 31v and tube lens was 125v. For the negative mode, the source voltage was 5Kv, capillary
11 voltage was -35v and tube lens was -90v.

12 The MS was operated using a Data-dependent acquisition (DDA) method. In brief, an
13 MS1 scan was performed using the Orbitrap detector scanning from 85 to 1000 m/z at a
14 resolution of 30,000 storing data in profile. Phthalate (413.266230 m/z) was used as lock-mass.
15 Then, MS2 (fragmentation event) was triggered on the most dominant ion found in the MS1
16 scan. This MS2 was performed in the ion trap, using collision-induced dissociation (CID) and
17 the data was stored as centroid.

18 Data was analysed using Qual Browser (Xcalibur 2.1) Thermo Scientific. Theoretically,
19 m/z was calculated for both the protonated (positive ion mode) and deprotonated (negative ion
20 mode) for each compound. Extracted ion chromatograms (EICs) for these m/z (5ppm mass
21 tolerance) as well as the UV chromatograms were generated at 280nm, 320nm and 520nm. The
22 retention time of the standards from the MS1 scans and the MS2 fragmentation spectra from
23 the standards were compared to the samples (unit resolution mass tolerance).

1 When the retention time, parent mass and fragmentation matched the standard, a
2 confident match was determined. In some instances, due to the nature of DDA experiments,
3 the ion of interest was not fragmented in which case only the retention time and parent mass
4 could be used and a less confident match was determined. In the case of phenolics, when there
5 were no standards and hence no retention time available, the fragmentation spectra were
6 referred solely on the match of fragmentation spectra reported in Kammerer et al. (22).

7

8 **Separation with Colloidal Gas Aphrons (CGA) using 10mM TWEEN20**

9 In the previous work by our group, it was found that high recovery of polyphenols from
10 grape ethanolic extracts could be obtained by CGA generated with the cationic surfactant Cetyl
11 trimethylammonium bromide (CTAB) and the non-ionic TWEEN20 (15). In the present work,
12 ethanolic and hot water extracts were first obtained from grape pomace (see Figure 1 for full
13 separation process). Hot water extract (HWE) was applied to the CGA for the first time. CGA
14 generated from 10mM TWEEN20 were then applied to each extract based on the optimum
15 conditions found in our previous work eg: the ratio of extract to the CGA was kept constant at
16 16:1 and the drainage time was kept at 5min. CGA separations of grape pomace extracts were
17 carried out in a flotation glass column (i.d 5cm, height: 50cm). The CGA were pumped by a
18 peristaltic pump (Watson Marlow) from the CGA generating container into the column which
19 contained 60mL of ethanolic extract of grape pomace. The volume of collapsed CGA and
20 drained liquid phase were measured. The initial extracts of EE and HWE contained 2624 mg
21 GAE_{TPI}/L and 1562 mg GAE_{TPI}/L respectively. Both fractions were diluted at an appropriate
22 dilution with deionized water for all the tests.

23 The percentage recovery of a specific compound (y) in the CGA phase (R_y) was
24 calculated based on the differences between the total amount of added y in the feed ($M_{y/feed}$)
25 and the amount of y measured in the separated liquid phase ($M_{y/liq}$). For some experiments, the

1 amount of y in the CGA phase was also calculated and the mass balance deviation was within
2 10%. The separation factor (SF) was also calculated based on the concentrations of compound
3 y in the CGA phase ($[y]_{CGA}$) and in the liquid phase ($[y]_{LP}$) as described in Eq.1:

$$SF = \frac{[y]_{CGA}}{[y]_{LP}} \quad (\text{Eq. 1})$$

7 **Collagenase and Elastase inhibitory activity of crude extracts and CGA fractions**

8 The inhibitory activity of gallic acid, grape pomace crude extracts and the CGA fractions
9 against *C. histolyticum* collagenase (ChC) and porcine pancreatic elastase (PPE) were
10 measured spectrophotometrically according to the method used by Wittenauer et al., (10) by
11 using a multi-mode Tecan GENios microplate reader equipped with analysis software Xfluo4
12 version 4.51 (Salzburg, Austria). Both enzymes were incubated with the extracts and their CGA
13 fractions with relevant substrates (see below). The inhibitory potential of the grape pomace
14 extracts were examined in dilutions so as to establish a dose-dependent curve in order to
15 calculate the half-maximal inhibitory concentrations (IC_{50}). Due to the high concentration of
16 polyphenols in the grape pomace extracts, the dilutions of 1:50 to 1:200 with total polyphenolic
17 contents ranging from 52.5 to 7.8 mg GAE/L were applied before being incubated with
18 collagenase and elastase.

19 **Collagenase (ChC) assay:** In this assay the enzymatic reaction rate was measured based on
20 the consumption of the substrate peptide FALGPA. Therefore, the slopes of the reaction rates
21 decreased with the increased in extract (inhibitor) concentration. Briefly, ChC (0.16 U/mL) and
22 FALGPA (3mM) were dissolved in 0.05M tricine buffer containing 0.4M NaCl and 0.01M
23 $CaCl_2$; the pH was adjusted to 7.5 with 1M NaOH. The inhibitory activity of the following
24 samples were measured:

- 1 a. Dilutions of ethanolic and hot water grape pomace extracts with water at concentration
2 of (1:50), (1:100) and (1:200) (extract:water).
- 3 b. CGA and liquid fractions derived from CGA separations generated from TWEEN20
4 surfactant.
- 5 c. Aqueous solution of gallic acid (43 mg/L, 85 mg/L, 128 mg/L and 170 mg/L).

6 Briefly, 30 μ l of the samples (a-c) were incubated with 10 μ l of ChC solution and 60 μ l
7 of tricine buffer for 20 mins at 37°C, after which, 20 μ l of FALGPA solution was added to
8 initiate the reaction. The reaction rate was measured over 20mins by measuring the decreased
9 in the absorbance of FALGPA at 340nm. Initial velocities were determined and a dose-
10 dependent curve was established. The concentration to inhibit 50% of the enzyme activity, IC₅₀
11 values were then determined from the curves. The inhibition activity (%) was calculated
12 according to Eq.2.

13
$$ChC \text{ inhibition } (\%) = \frac{Initial \text{ velocity } control - Initial \text{ velocity } sample}{Initial \text{ velocity } control} * 100 \quad (Eq.2)$$

14

15

16 **Elastase (PPE) assay:** porcine pancreatic elastase (PPE) inhibitory activity of the individual
17 samples (a-c) was determined spectrophotometrically by using the AAAPVN as the substrate
18 and by monitoring the production of p-nitroaniline at 405nm to determine the reaction rate.
19 Briefly, 10 μ l was taken and loaded into wells together with 100 μ l of Tris buffer and 30 μ l of
20 samples. The mixture was incubated for 20mins at 25°C. Subsequently, 40 μ l of the AAAPVN
21 (dissolved in 2mM Tris buffer at 0.25mg/mL) was added. Since the PPE was performed with
22 AAAPVN as the substrate peptide, the enzyme activity can be calculated from the released of
23 p-nitroaniline as a product, leading to the increased in absorption values. The absorbance was

1 monitored for 20mins after the addition of AAAPVN and the initial velocities, the inhibitory
2 effect and IC₅₀. The values were calculated as in Eq.2.

3

4 **Statistical analysis**

5 All the experiments were performed in triplicate. The data were subjected to the
6 analysis of variance using IBM® SPSS® Statistics 21 software programme where statistical
7 differences were noted. Differences among different treatments were determined using Tukey
8 test. The significance level was defined at $p < 0.05$. The results were reported as means \pm SD.

9

10 **RESULTS AND DISCUSSIONS**

11 **Ethanollic and hot water extraction**

12

13 The chemical composition of the crude grape pomace extracts was determined. In
14 general, the content of total phenols, anthocyanin and protein was higher in EE but sugar was
15 higher in HWE. The total phenolic content in EE was 21.0 ± 0.1 mg GAE/g of pomace. This
16 value was almost two times higher than in HWE (12.5 ± 0.1 mg GAE/g pomace). A similar
17 result was obtained by the Folin-Ciocalteu method where EE had 22.0 ± 0.2 mg GAE/g while
18 HWE had 17.0 ± 0.2 mg GAE/g dry weight pomace. These results closely followed the values
19 obtained in the literature for grape pomace (17,23) and they were higher than those obtained
20 for the Brazilian grape extract as reported by Beres et al. (24). The total monomeric
21 anthocyanins content in EE was 6.6 ± 0.6 mg ME/g, almost three times higher than in HWE
22 (2.3 ± 0.7 mg ME/g dry weight). Low levels of protein were recovered in both extracts (0.4 and
23 0.2 mg BSA equivalent/g dry weight of grape pomace) and a slightly higher sugar was extracted
24 in HWE than in EE.

25

1 **Polyphenol composition of crude grape pomace extracts**

2 The main composition of the EE and HWE analysed by HPLC is shown in Table 1.
3 Qualitative analysis with LC-MS was also conducted to confirm the identification and/or
4 identify the individual polyphenols in EE and HWE. It must be noted that minor amounts of
5 phenolics may escape from the extraction due to the interaction with dietary fibers, proteins
6 and other polymerised structures (22). In this analysis, fourteen standards of phenols and
7 anthocyanins were analysed against both extracts as not all standards were commercially
8 available. Retention time of standards, MS1 spectra and MS2 fragmentation spectra of the
9 standards were compared to samples'. If the retention time, MS1 and MS2 matched, a confident
10 assignment was given. If only the retention time and MS1 matched, a semi-confident
11 assignment was given. The results of the mass spectrometry data in both positive-ion mode
12 (anthocyanins) and negative-ion mode (phenolic acids, anthoxanthins, stilbenes, flavonols and
13 flavanols) of compounds in the extracts are shown in Table 2.

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1 Table 1: Polyphenol contents (mg/L) of grape pomace extracts and CGA fractions.

Concentration (mg/L)						
Compound/Sample	EE	CGA	LP	HWE	CGA	LP
<i>Phenolic acids</i>						
Gallic acid	32.3 ± 2.8	4.1 ± 0.1	2.2 ± 0.3	74.5 ± 6.8	24.9 ± 1.4	12.2 ± 2.5
Caffeic acid	17.5 ± 1.4	ND	ND	12.4 ± 0.3	ND	ND
Syringic acid	24.5 ± 0.9	2.5 ± 0.1	2.0 ± 0.1	17.6 ± 0.4	10.2 ± 3.9	7.4 ± 1.8
Chlorogenic acid	7.1 ± 0.4	ND	ND	21.2 ± 2.3	ND	3.5 ± 2.5
4-hydroxy benzoic acid	ND	ND	ND	6.9 ± 0.1	ND	ND
Total phenol acids	81.3 ± 5.3	6.6 ± 0.1	4.2 ± 0.1	132.6 ± 9.1	35.1 ± 5.3	23.1 ± 6.8
<i>Flavonols</i>						
Resveratrol	36.80 ± 3.90	ND	ND	ND	ND	ND
Quercetin	108.40 ± 5.10	31.1 ± 0.5	17.4 ± 0.7	29.6 ± 0.3	ND	ND
Kaempferol	16.10 ± 1.50	6.5 ± 0.2	3.9 ± 0.7	67.5 ± 0.9	23.5 ± 0.1	24.4 ± 0.1
Total flavonols	161.30 ± 10.50	37.6 ± 0.7	21.3 ± 1.4	97.1 ± 1.1	23.5 ± 0.1	24.4 ± 0.1
<i>Flavanols</i>						
Catechin	3.1 ± 0.2	ND	ND	30.9 ± 0.1	9.5 ± 0.3	ND
Epicatechin	28.7 ± 4.0	ND	ND	18.3 ± 0.6	ND	ND
Total flavanols	31.8 ± 4.2	ND	ND	49.2 ± 0.6	9.5 ± 0.3	ND
<i>Anthocyanins</i>						
Delphinidin 3-o-glucoside	72.2 ± 15.5	36.0 ± 6.3	17.6 ± 7.8	29.7 ± 0.2	1.9 ± 0.6	1.0 ± 0.9
Petunidin 3-o-glucoside	33.5 ± 16.3	17.4 ± 7.5	9.4 ± 0.3	11.6 ± 0.4	1.1 ± 0.4	0.7 ± 0.5
Cyanidin 3-o-glucoside	13.8 ± 2.1	8.2 ± 0.3	4.0 ± 0.2	4.9 ± 0.1	0.2 ± 0.3	0.1 ± 0.2
Malvidin 3-o-glucoside	85.0 ± 17.2	41.2 ± 0.1	23.9 ± 0.3	30.2 ± 0.1	3.7 ± 1.5	2.4 ± 1.3
Total anthocyanins	204.5 ± 51.1	102.8 ± 13.9	54.9 ± 7.9	76.5 ± 0.5	7.0 ± 2.6	4.2 ± 2.8
Total	478.9 ± 71.0	147.0 ± 14.8	80.44 ± 9.33	355.2 ± 11.4	75.0 ± 8.0	51.7 ± 9.6

2 ND: not detected; EE: ethanol extract; HWE: hot water extract; CGA: CGA phase; LP: liquid phase.

3 Values represent mean ± standard deviation (n = 3).

4

5

6

7

1 Table 2: LC-MS data of phenolic compounds extracted from grape pomace

No.	Compound	Retention time (min)	m/z	MS/MS fragments m/z	EE	HWE
Phenolic acids						
[M-H]-						
1	* Gallic acid	3.2	169.0142	125	√	√
2	Caftaric acid	6.5	311.0409	179/135		√
3	*p-hydroxybenzoic acid	7.3	137.0244	93	√	√
4	* Caffeic acid	10.6	179.0350	135	√	√
5	* Ferulic acid	10.8	193.0506	134	√	√
6	* Fertaric acid	10.9	325.0565	193	√	√
7	Syringic acid	11.4	197.0455	153/182	√	√
Anthoxanthins and Stilbenes						
[M-H]-						
8	Procyanidin B1	7.4	577.1351	407/425	√	√
9	* Catechin	8.9	289.0718	245	√	√
10	* Procyanidin B2	10.1	577.1351	407/425	√	√
11	* Epicatechin	13.1	289.0718	245	√	√
12	* Epicatechin gallate	19.6	441.0827	289		√
13	* <i>trans</i> -resveratrol	23.1	227.0714	185	√	√
14	* Quercetin	20.4	301.0354	151/179	√	√
15	Kaempferol	27.9	285.0405	257	√	√
16	Quercetin 3-o-galactoside	19.8	463.0882	301	√	√
17	Quercetin 3-o-glucoside	20.4	463.0882	301	√	√
Anthocyanins						
[M]+						
18	*Delphinidin 3-o-glucoside	8.52	465.1028	303	√	√
19	*Cyanidin 3-o-glucoside	10.9	449.1078	287	√	√
20	*Petunidin 3-o-glucoside	12.4	479.1184	317	√	√
21	*Malvidin 3-o-glucoside	14.1	493.1341	331	√	√
22	Peonidin 3-o-glucoside	14.7	463.1235	301	√	√
23	Delphinidin 3-o-acetylglucoside	16.3	507.1133	303	√	√
24	Cyanidin 3-o-acetylglucoside	18.7	491.1184	287	√	√
25	Malvidin 3-o-acetylglucoside	21.5	535.1446	331	√	√
26	Peonidin 3-o-acetylglucoside	21.6	505.1341	301	√	√

27	Cyanidin 3-o-p-coumaroylglucoside	23.1	595.1446	287	√	√
28	Petunidin 3-o-p-coumaroylglucoside	23.9	625.1552	317	√	√
29	Peonidin 3-o-p-coumaroylglucoside	25.1	609.1603	301	√	√
30	Malvidin 3-o-p-coumaroylglucoside	25.6	639.1708	331	√	√

1 All compounds were confirmed according to Kammerrer et al. (22).

2 *compounds were confirmed with pure standards.

3 EE: ethanol extract; HWE: hot water extract.

4

5 A total of 30 phenolic compounds were present in both extracts. Among these, 7 phenolic
6 acids, 10 anthoxanthins and stilbenes and 13 anthocyanins were detected in both extracts. All
7 anthocyanins detected were of monoglucoside (glu), acetyl and p-coumaroyl derivatives of
8 delphinidin (DEL), cyanidin (CYA), petunidin (PET), peonidin (PEO) and malvidin (MAL).
9 Out of these 30 compounds, 15 were given confident assignment as the retention times, MS1
10 and MS2 matched with the standards. These compounds were gallic acid, p-hydroxybenzoic
11 acid, caffeic acid, ferulic acid, fertaric acid, catechin, procyanidin B2, epicatechin, epicatechin
12 gallate, trans-resveratrol, quercetin, delphinidin 3-o-glucoside, cyanidin 3-o-glucoside,
13 petunidin 3-o-glucoside and malvidin 3-o-glucoside. The MS2 mode was used to provide
14 information on the aglycone and its corresponding sugar due to the observed m/z fragmentation
15 values (303 for DEL; 287 for CYA; 317 for PET; 301 for PEO; and 331 for MAL) which were
16 matched to those reported in the literature (22). In this analysis, quercetin 3-o-glucoside and
17 quercetin 3-o-galactoside have the same MS1 and MS2, therefore their retention times are the
18 same; thus, differentiation of these polyphenols cannot be made. In the case of anthocyanins,
19 all anthocyanins and derivatives were present in both EE and HWE. However, differences were
20 noted in the composition of phenolic acids and anthoxanthins/stilbenes between both extracts
21 where the EE was lacking the presence of caftaric acid and epicatechin gallate.

22 In general, both extracts had the same type of compounds present but interestingly they
23 differed in their composition. This is particularly clear when the mass percentage of groups of

1 polyphenols (eg: phenolic acids) is calculated from data in Table 1. For example, phenolic
2 acids were present at higher proportion in the HWE (37%) than in the EE (17%); in both
3 extracts gallic acid was the predominant phenolic acid. Similarly flavanols were at higher
4 proportion in HWE (14%) than in EE (7%). However the composition of flavonols was similar
5 in both extracts, (34% and 27% in EE and HWE respectively) but quercetin was predominant
6 in EE and Keampferol in HWE. The anthocyanins composition was higher in EE (43%), than
7 in HWE (22%). However, with regards to the collagenase and elastase inhibitory activities and
8 their relationship to polyphenols composition (see below) phenolic acids, flavonols and
9 flavanols were the most relevant as anthocyanins have not been related to these activities.

10

11 **Separation of polyphenols from crude grape extracts by CGA**

12 Table 3 shows the recovery (%) and separation factor (SF) of the CGA separation from
13 EE and HWE. Very similar recoveries of phenols and anthocyanins were obtained from both
14 extracts. Generally, the recovery of compounds was higher in EE than in HWE. A separation
15 factor higher than one indicated higher affinity of the compound for the CGA phase than the
16 liquid phase. This was the case for all compounds in both extracts although higher SF's were
17 obtained for EE. The selectivity of the separation in relation to both protein and sugar was low
18 as these were also preferentially separated into the CGA phase although the SF of sugar from
19 HWE was lower than one. The low ratio value of V_{LP}/V_{CGA} (ie: low volume of liquid drained
20 in relation to volume of CGA) was an indication of a stable CGA which might be due to the
21 presence of other compounds (glucose and proteins) which could increase the viscosity of the
22 liquid in the continuous phase and hence increased the stability of the CGA (15). It is also
23 important to highlight that some aggregates were observed in the CGA phase which did not
24 completely solubilise during analysis, hence this would probably lead to an underestimation of
25 the net recovery. Overall, the recovery results were in agreement with our previous work (15).

1

2 Table 3: Recovery efficiency (%) and separation factor (SF) by CGA separations of EE and HWE

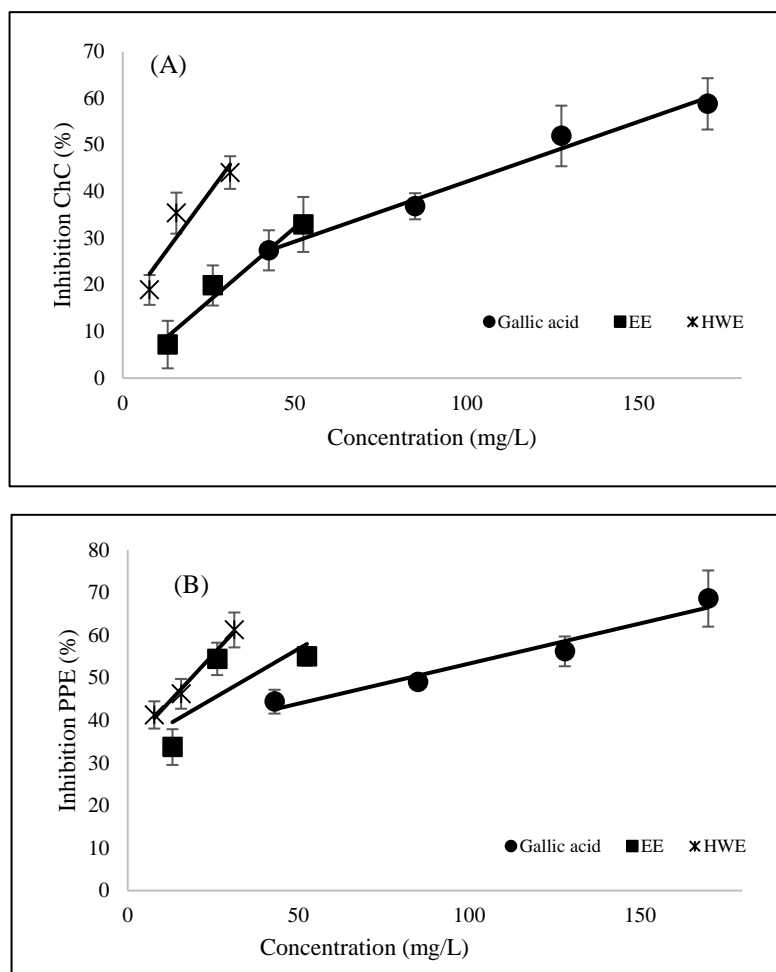
Extract	EE	HWE
V_{CGA}/V_{feed}	16	16
$V_{LP}/V_{CGA-phase}$	0.50	0.55
Recovery (%)		
GAE _{FI}	83.45 ^{bc}	85.87 ^c
GAE _{TPI}	79.40 ^b	71.39 ^{ab}
ME	84.99 ^c	77.39 ^b
Glucose	71.74 ^a	68.91 ^a
Protein	85.86 ^c	66.45 ^a
SF		
GAE _{FI}	4.71 ^c	1.31 ^b
GAE _{TPI}	1.89 ^b	1.20 ^b
ME	1.47 ^a	1.31 ^b
Glucose	1.65 ^a	1.34 ^b
Protein	1.72 ^{ab}	0.87 ^a

3 GAE_{FI}, Gallic acid equivalent (Folin-Cioulcateau index; GAE_{TPI}, Gallic acid equivalent (total phenol index); ME,
4 Malvidin glucoside equivalent; $V_{LP}/V_{CGA-phase}$, ratio of volume of liquid phase to the volume of CGA phase. Same
5 superscript letters in the same column (for each recovery and SF) indicates means were not statistically different
6 ($p>0.05$) according to ANOVA (n=3).

7

8 **Collagenase and elastase inhibitory activity**

9 The ethanolic (EE) and hot water extracts (HWE) of grape pomace were tested for their
10 ChC and PPE inhibitory activity. Collagen, which occupies around 70-80% of the skin weight
11 is known to provide structural integrity (6). Due to skin ageing, collagen is rapidly degraded
12 by the action of collagenase. As shown in Figures 2(A) and 2(B), the grape pomace extracts
13 showed a linear dose-dependant relationship with inhibitory activities. From these dose-
14 dependent relationships, IC₅₀ values were calculated to be 35.4mg/L (HWE), 78.8mg/L (EE)
15 and 130mg/L (gallic acid). The maximum inhibitory activity measured for EE was 34%,
16 therefore above this activity (up to 50%) a linear relationship with concentration was assumed
17 in order to determine the IC₅₀.



1 Figure 2: Dose dependent inhibition of collagenase (A) and elastase (B) activity by gallic acid, ethanol
 2 extract (EE) and hot water extract (HWE) (n =3).

3

4 The same trend was observed for PPE inhibitory activity. Elastin is an insoluble fibrous
 5 protein which occupies only 2-4% of the skin dermis weight but plays a vital role ensuring the
 6 elasticity of the skin (6). Based on the IC₅₀ results, HWE (18.7mg/L) had the highest potency
 7 as compared to EE (35.5mg/L) and gallic acid (82.0mg/L). Similar IC₅₀ value was obtained with
 8 the methanolic extract of grape pomace (14.7mg/L) which may suggests comparable
 9 polyphenol composition (10).

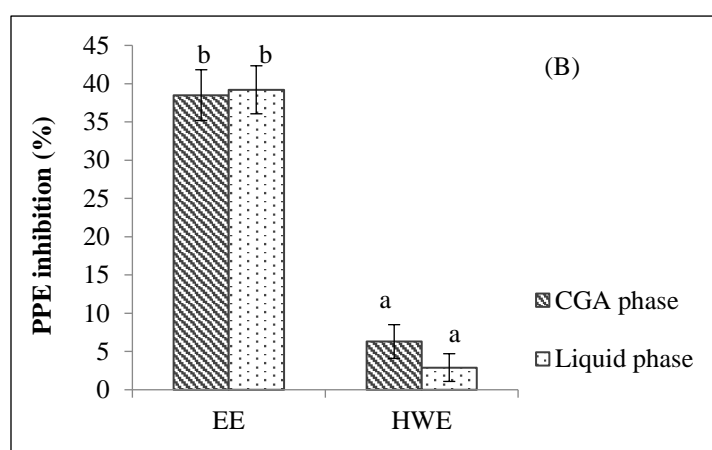
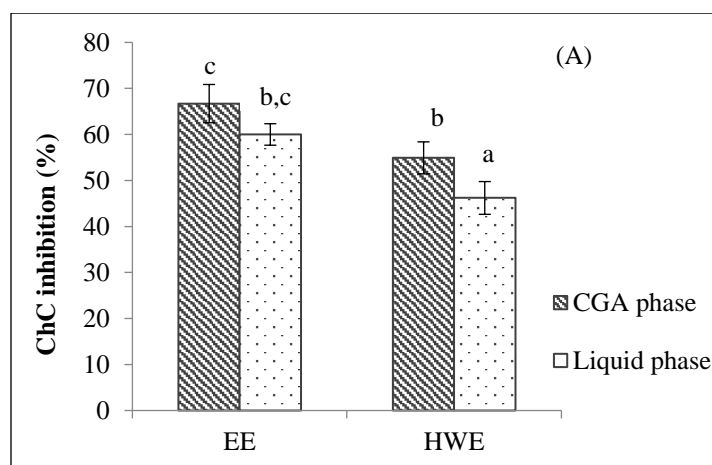
10 The higher inhibitory activity of HWE than EE against collagenase and elastase can be
 11 explained by the differences in polyphenols composition. The phenolic acids such as gallic acid
 12 and chlorogenic acid in HWE which account for 37% of total polyphenols could have a

1 pronounced effect on the inhibitory activities. Gallic acid, a low molecular weight hydrophilic
2 compound could play an important part in the observed activity by accessing the active centre
3 site of the elastase and blocking the binding of substrates to this site (10). However, given that
4 the potency of the extract was superior than that of the gallic acid alone (Table 4), it is clear
5 that other components also may contribute to the activity, perhaps in a synergistic manner.
6 Chlorogenic acid, for example, which is a derivative of cinnamic acid, could also contribute as
7 it is well known for its potent antioxidant and anti-inflammatory activities (25). Moreover, the
8 catechin and epicatechin which were present at high proportion in HWE (14%) could interact
9 with the elastase by hydrophobic interactions, causing conformational changes of elastase and
10 thus increasing the inhibitory activity (7). On the other hand, EE had high composition of
11 flavonols, particularly quercetin and resveratrol but they are larger molecules with lower
12 solubility in water than the phenolic acids which could possibly limit their activity.

13

14 **Collagenase and elastase inhibitory activity of CGA fractions in relation to polyphenolic** 15 **profile**

16 In order to determine the most active fractions after separation by CGA, CGA and liquid
17 phases from both EE and HWE were tested for ChC and PPE. The inhibitory activities against
18 ChC and PPE are shown in Figures 3(A) and (B), respectively. Contrary to the crude extracts,
19 EE fractions demonstrated higher activity than HWE fractions, CGA-EE had 67% collagenase
20 inhibitory activity and CGA-HWE 55%; the liquid phases had 60% and 46% activity,
21 respectively. This small difference in activity between the liquid and CGA phases can be
22 explained based on their polyphenol composition (Table 1).



1 Figure 3: Anti-collagenase activity (A) and anti-elastase activity (B) of CGA fractions from EE and
 2 HWE. Bars are means \pm standard deviation of three determinations ($n = 3$). Same superscript letters
 3 indicates means with no significant difference ($p > 0.05$) according to ANOVA ($n=3$).

4

5 For example, the composition of phenolic acids in CGA-HWE and LP-HWE were almost
 6 the same (mass percentages of phenolic acids over total phenols were 47% and 45%
 7 respectively) and for flavonols composition was higher in the liquid phase (31% in CGA and
 8 47% in liquid phase). The same trend was noted in CGA-EE and LP-EE where phenolic acids
 9 and flavonols composition was very similar in both fractions (4.5 and 5.2% phenolic acids in
 10 CGA and LP respectively and 26% flavonols in both fractions). This similarity in composition
 11 supports the insignificant differences in inhibitory activities of these fractions against both
 12 enzymes. Kaempferol was found at high concentration in both CGA-HWE and LP-HWE (23.5
 13 and 24.4 mg/L respectively) but in the case of CGA-EE, the most predominant flavonol was

1 quercetin (31.1mg/L). These compounds could possibly be the main contributors to the
 2 inhibitory activities observed whereby the hydroxyl group in C-3 might played a role in
 3 conferring the inhibitory activity (9). Moreover, the high content of gallic acid in CGA-HWE
 4 (24.9mg/L) and in LP-HWE (12.2mg/L) could also be important for the ChC inhibitory
 5 activity. The hydroxyl group from gallic acid could act as a hydrogen bond acceptor/donors
 6 with the hydroxyl, amino or carboxyl groups of the collagenase's side chain functional groups
 7 which can alter its structure, while the benzene rings of the polyphenols can form hydrophobic
 8 interactions with collagenase (8,10).

9 The differences in inhibitory activity against PPE between the LP and CGA fractions (Fig 3B)
 10 could be explained based on the differences in composition (see above). On the other hand the
 11 much higher activity in the EE fractions than in the HWE fractions could not be clearly
 12 explained in terms of differences in composition of groups of polyphenols but individual
 13 polyphenols. For example, quercetin was predominantly present in the CGA-EE whilst none
 14 was detected in the CGA-HWE. This suggests that quercetin is a key compound responsible
 15 for PPE inhibition. Quercetin could possibly alter the specificity of the elastase substrate by
 16 interacting with subsite of MMP-9 active site (26).

17 Table 4: Inhibitory efficiency (%/mg GAE_{Fl}L⁻¹) of crude extracts and CGA-separated fractions

Extract/Activity	EE	CGA-EE	LP-EE	HWE	CGA-HWE	LP-HWE	Gallic acid
ChC	0.63	2.43	2.18	1.41	2.45	0.37	0.38
PPE	1.41	1.40	1.43	2.67	0.28	0.02	0.61

18

19

20 In order to assess if any of the fractions had been preferentially enriched with the most
 21 active polyphenols the activity potency had to be determined. However, these fractions showed
 22 poor dose-dependency relationship (data not shown) and the IC₅₀ could not be determined.

1 Therefore, the inhibitory potency of CGA fractions was expressed as inhibitory efficiency
2 which is the activity in relation to the total phenols content (% / mg GAE_{FL}L⁻¹) (Table 4). The
3 CGA-EE fraction was found to be about four times more efficient than its crude extract and
4 over six times more efficient than gallic acid in relation to ChC inhibitory activity. However
5 the efficiency of both CGA and LP fractions was almost the same which is in agreement with
6 results in Fig 3. Interestingly the efficiency in CGA-HWE was seven times higher than in LP
7 and almost double that in the raw extract (HWE). Moreover the efficiency of the CGA
8 fractions of both raw extracts was six times higher than gallic acid's which suggests that the
9 inhibition of these enzymes could be the result of synergistic activity of different polyphenols.
10 This has been observed in a formulation of four combined super fruits extract (*Ginkgo biloba*,
11 *Punica granatum*, *Ficus carica*, and *Morus alba*) against collagenase (27).

12 In the case of PPE inhibitory activity, no increase in efficiency was noted for the
13 CGA/LP fractions of EE and the efficiency of the HWE decreased after CGA separation. The
14 inhibitory efficiencies of the raw extracts were superior to that of pure gallic acid.

15 From results above it could be hypothesised that TWEEN20 might play a role in
16 facilitating the delivery of the polyphenols to the target site of the collagenase. This explained
17 why the efficiency of the CGA-EE increased substantially as compared to the crude extract's
18 and it was comparable to that of CGA-HWE. It is also worth mentioning that the surfactant did
19 not inhibit or activate both ChC and PPE (data not shown) hence, the inhibitory activities were
20 solely due to the action of polyphenols in the fractions. Non-ionic surfactants were known to
21 cause the least irritating effect to skin compared to anionic surfactants hence they were
22 preferred for inclusion in many skin care products (28). Moreover, surfactants in general are
23 known to alter the skin permeation by forming non-specific hydrophobic interactions involving
24 the alkyl chains of the surfactant and the hydrophobic regions of the keratin in stratum corneum
25 (30). Most studies about non-ionic surfactants and biological activities revealed that the C12

1 alkyl chain was the most important character in terms of perturbation of the membrane which
2 explained the surfactant solubility and partitioning (31). Although most studies revealed that
3 their interactions with non-ionic surfactants did not alter skin permeation to a significant level,
4 enhancement has been noted in some studies whereby penetration of lidocaine (a type of drug)
5 significantly increased through hairless mouse skin with TWEEN20 and TWEEN60 (28).

6

7 **CONCLUSIONS**

8 The extraction of polyphenols from grape pomace by ethanol and hot water led to crude
9 extracts with different polyphenol compositions and this also resulted in differences in
10 collagenase and elastase inhibitory activity. Phenolic acids were present at higher proportion
11 in the HWE (37%) than in the EE (17%) which suggested their important role in the inhibition.
12 HWE was the most efficient at inhibiting both collagenase and elastase and both EE and HWE
13 were superior to gallic acid. Further extraction by CGA led to higher inhibitory efficiency
14 against collagenase although there was no difference in efficiency between the separated
15 phases for EE but there was for HWE. Gallic acid was the main component of the CGA-HWE
16 but other polyphenols (eg: kaempferol) must have contributed also to its potency as this fraction
17 inhibited collagenase over six times more efficiently than gallic acid. The CGA-EE fraction
18 was found to be about four times more efficient than its crude extract and over six times more
19 efficient than gallic acid in collagenase's inhibition; quercetin was found to be the major
20 polyphenol in this fraction. These results suggested that although quercetin was highly
21 insoluble in water and had high molecular weight, TWEEN20 helped to improve its solubility
22 and therefore facilitated its delivery to the enzyme. Therefore, CGA separation led to fractions
23 enriched in active polyphenols with enhanced collagenase inhibitory activity in both CGA and
24 liquid phases. Although the polyphenols composition in CGA and liquid phases in both extracts

1 were very similar, and hence their inhibitory activities, it must be stressed that further
2 separation with CGA led to CGA fractions with less sugar and protein (and ethanol when
3 applied to the ethanolic extract) which can be an advantage in terms of formulation. It should
4 be noted that the concentration of these polyphenols in the CGA fractions were topically
5 relevant (generally between 25-100 μ M). Moreover the surfactant in these fractions could act
6 as a carrier and solubilising agent to enhance the permeation of polyphenols across the skin.
7 Therefore, the surfactant rich solution may provide an optimum media that could facilitate the
8 permeation of the polyphenols through the skin. This research shows the potential of CGA to
9 revalorise the grape marc and to obtain an extract with potential in cosmetics applications.

10

11

12 REFERENCES

- 13 1. Mena F, Mena A, Tréton J. Polyphenols against Skin Aging. *Polyphenols Hum Heal*
14 *Dis.* 2013;1:819–30.
- 15 2. Haslam E, Cai Y. Plant polyphenols (vegetable tannins): gallic acid metabolism. *Nat*
16 *Prod Rep.* 1994;11(1):41–66.
- 17 3. Perona JS, Cabello-Moruno R, Ruiz-Gutierrez V. The role of virgin olive oil
18 components in the modulation of endothelial function. *J Nutr Biochem* [Internet].
19 2006;17(7):429–45. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16481154>
- 20 4. Chiu AE, Chan JL, Kern DG, Kohler S, Rehmus WE, Kimball AB. Double-blinded,
21 placebo-controlled trial of green tea extracts in the clinical and histologic appearance of
22 photoaging skin. *Dermatol Surg* [Internet]. 2005;31(7 Pt 2):855–60; discussion 860.
23 Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16029678>
- 24 5. Türkoğlu M, Uğurlu T, Gedik G, Yılmaz AM, Süha Yalçın A. In vivo evaluation of
25 black and green tea dermal products against UV radiation. *Drug Discov Ther* [Internet].
26 2010;4(5):362–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22491240>
- 27 6. Hong Y-H, Jung EY, Noh DO, Suh HJ. Physiological effects of formulation containing
28 tannase-converted green tea extract on skin care: physical stability, collagenase,
29 elastase, and tyrosinase activities. *Integr Med Res* [Internet]. Korea Institute of Oriental
30 Medicine; 2014;3(1):25–33. Available from:
31 <http://www.sciencedirect.com/science/article/pii/S2213422013000991>
- 32 7. Wahab NA, Rahman RA, Ismail A, Mustafa S, Hashim P. Assessment of Antioxidant
33 Capacity, Anti-collagenase and Anti-elastase Assays of Malaysian Unfermented Cocoa
34 Bean for Cosmetic Application. *Nat Prod Chem Res* [Internet]. 2014;2(2). Available
35 from: <http://dx.doi.org/10.4172/2329-6836.1000132>

- 1 8. Madhan B, Krishnamoorthy G, Rao JR, Nair BU. Role of green tea polyphenols in the
2 inhibition of collagenolytic activity by collagenase. *Int J Biol Macromol.*
3 2007;41(1):16–22.
- 4 9. Sin BY, Kim HP. Inhibition of Collagenase by Naturally-Occurring Flavonoids. *Arch*
5 *Pharm Res* [Internet]. 2005;28(10):1152–5. Available from: <http://apr.psk.or.kr>
- 6 10. Wittenauer J, Mäckle S, Sußmann D, Schweiggert-weisz U, Carle R. Inhibitory effects
7 of polyphenols from grape pomace extract on collagenase and elastase activity.
8 *Fitoterapia* [Internet]. Elsevier B.V.; 2015;101:1–9. Available from:
9 <http://dx.doi.org/10.1016/j.fitote.2015.01.005>
- 10 11. Zillich O V, Schweiggert-Weisz U, Hasenkopf K, Eisner P, Kersch M. Release and in
11 vitro skin permeation of polyphenols from cosmetic emulsions. *Int J Cosmet Sci*
12 [Internet]. 2013;35(5):491–501. Available from: <http://dx.doi.org/10.1111/ics.12072>
- 13 12. Löf D, Schillèn K, Nilsson L. Flavonoids: Precipitation kinetics and interaction with
14 surfactant micelles. *J Food Sci.* 2011;76(3):35–9.
- 15 13. Yutani R, Morita S, Teraoka R, Kitagawa S. Distribution of Polyphenols and a
16 Surfactant Component in Skin during Aerosol OT Microemulsion-Enhanced
17 Intradermal Delivery. *Chem Pharm Bull.* 2012;60(8):989–94.
- 18 14. Williams AC, Barry BW. Penetration enhancers. *Adv Drug Deliv Rev.* 2004;56(5):603–
19 18.
- 20 15. Spigno G, Amendola D, Dahmoune F, Jauregi P. Colloidal gas aphrons based separation
21 process for the purification and fractionation of natural phenolic extracts. *Food Bioprod*
22 *Process* [Internet]. Institution of Chemical Engineers; 2015;94:434–42. Available from:
23 <http://dx.doi.org/10.1016/j.fbp.2014.06.002>
- 24 16. Spigno G, Jauregi P. Recovery of Gallic Acid with Colloidal Gas Aphrons (CGA). *Int J*
25 *Food Eng* [Internet]. 2005;1(4):1–10. Available from:
26 [http://www.degruyter.com/view/j/ijfe.2005.1.4/ijfe.2005.1.4.1038/ijfe.2005.1.4.1038.x](http://www.degruyter.com/view/j/ijfe.2005.1.4/ijfe.2005.1.4.1038/ijfe.2005.1.4.1038.xml)
27 [ml](http://www.degruyter.com/view/j/ijfe.2005.1.4/ijfe.2005.1.4.1038/ijfe.2005.1.4.1038.xml)
- 28 17. Amendola D, De Faveri DM, Spigno G. Grape marc phenolics: Extraction kinetics,
29 quality and stability of extracts. *J Food Eng.* 2010;97:384–92.
- 30 18. Singleton VL, Rossi JAJ. Colorimetry of total phenolics with acid reagents. *Am J Enol*
31 *Vitic.* 1965;16:144–58.
- 32 19. Lee J, Rennaker C, Wrolstad RE. Correlation of two anthocyanin quantification
33 methods: HPLC and spectrophotometric methods. *Food Chem.* 2008;110(3):782–6.
- 34 20. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al.
35 Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985;150:76–85.
- 36 21. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for
37 Determination of Sugars and Related Substances. *Anal Chem.* 1956;28(3):350–6.
- 38 22. Kammerer D, Claus A, Carle R, Schieber A. Polyphenol Screening of Pomace from Red
39 and White Grape Varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. *J Agric Food*
40 *Chem.* 2004;52:4360–7.
- 41 23. Tseng A, Zhao Y. Wine grape pomace as antioxidant dietary fibre for enhancing
42 nutritional value and improving storability of yogurt and salad dressing. *Food Chem.*

- 1 2013;138(1):356–65.
- 2 24. Beres C, Simas-Tosin FF, Cabezudo I, Freitas SP, Iacomini M, Mellinger-Silva C, et al.
3 Antioxidant dietary fibre recovery from Brazilian Pinot noir grape pomace. *Food Chem*
4 [Internet]. Elsevier Ltd; 2016;201:145–52. Available from:
5 <http://www.sciencedirect.com/science/article/pii/S0308814616300383>
- 6 25. Farah A, Monteiro M, Donangelo CM, Lafay S. Chlorogenic Acids from Green Coffee
7 Extract are Highly Bioavailable in Humans 1 , 2. *J Nutr.* 2008;(July):2309–15.
- 8 26. Saragusti AC, Ortega MG, Cabrera JL, Estrin DA, Marti MA, Chiabrando GA.
9 Inhibitory effect of quercetin on matrix metalloproteinase 9 activity Molecular
10 mechanism and structure-activity relationship of the flavonoid-enzyme interaction. *Eur*
11 *J Pharmacol* [Internet]. Elsevier B.V.; 2010;644(1–3):138–45. Available from:
12 <http://dx.doi.org/10.1016/j.ejphar.2010.07.001>
- 13 27. Ghimeray AK, Jung US, Lee HY, Kim YH, Ryu EK, Chang MS. In vitro antioxidant,
14 collagenase inhibition, and in vivo anti-wrinkle effects of combined formulation
15 containing *Punica granatum*, *Ginkgo biloba*, *Ficus carica*, and *Morus alba* fruits extract.
16 *Clin Cosmet Investig Dermatol.* 2015;
- 17 28. Zatz JL, Lee B. Skin Penetration Enhancement by Surfactants. In: Rieger MM, Rhein
18 LD, editors. 2nd ed. New York: Marcel Dekker; 1997. p. 501–17.

19

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