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

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

Abstract

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

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

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

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

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

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

INTRODUCTION

Over the past two decades, research on the use of natural products, particularly polyphenols, in beauty products has been active but challenging (1). Polyphenols with a hydroxyl group (-OH) attached to an aromatic benzene ring (C_6H_5-) naturally occur in plants and are therefore abundant in our diet (eg: vegetables, fruits, nuts, seeds and flowers), and have been extensively studied for their protective health effects against cardiovascular diseases and cancers (2). Moreover, they have been proven to exhibit significant antioxidant activity, as well as a UV protection effect which are very crucial for skin care products formulation (3).

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

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

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

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

MATERIALS AND METHODS

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

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

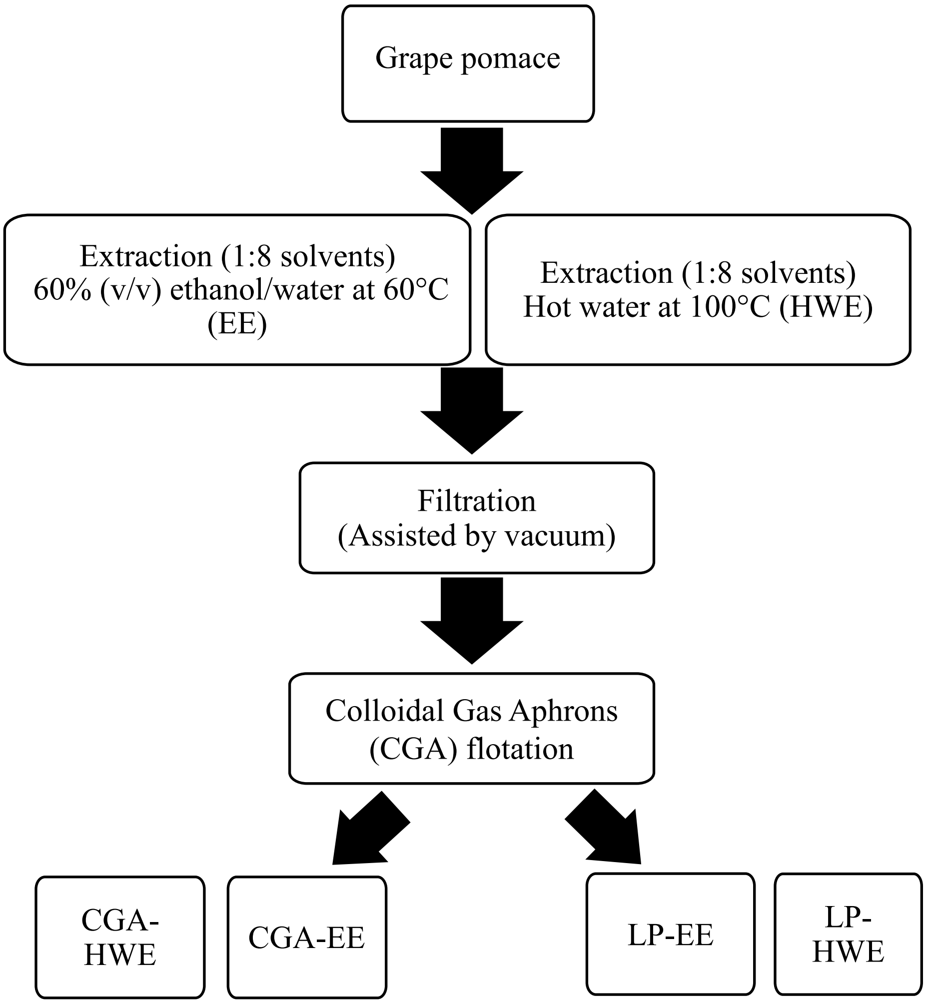

Figure 1: Schematic representation of the extraction of polyphenolic compounds present in grape pomace. The whole procedure was performed in triplicate (n = 3). EE, ethanol extract, HWE, hot water extract, CGA-EE; Aphron phase of EE, CGA-HWE; Aphron phase of HWE, LP-EE, liquid phase of EE and LP-HWE;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

Characterisation of grape pomace

Phenolic compounds

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

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

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

Non-phenolic compounds

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

Total sugar content was performed adopting the method from Dubois et al. (21). In test tubes, 0.4mL of glucose standard/sample was added followed by 0.2mL or 5% phenol solution. Subsequently, 1mL of sulphuric acid was pipetted direct to the solution and vortexed. The mixture was allowed to stand for 20-30mins to cool off. The absorbance of the mixture was read at 490nm and a calibration curve was constructed with different concentrations (10-100mg/L) of glucose standard. The results were expressed as mg/L glucose equivalent.

Determination of polyphenols composition by HPLC

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

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

Identification of polyphenols by LC-MS

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

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

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

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

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

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

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

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

The percentage recovery of a specific compound (y) in the CGA phase (Ry) was calculated based on the differences between the total amount of added y in the feed ($M_{y/\mathrm{feed}}$) and the amount of y measured in the separated liquid phase ($M_{y/\mathrm{liq}}$). For some experiments, the amount of *y* in the CGA phase was also calculated and the mass balance deviation was within 10%. The separation factor (SF) was also calculated based on the concentrations of compound y in the CGA phase ($[y]_{\text{CGA}}$) and in the liquid phase ($[y]_{\text{LP}}$) as described in Eq.1:

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

Collagenase and Elastase inhibitory activity of crude extracts and CGA fractions

The inhibitory activity of gallic acid, grape pomace crude extracts and the CGA fractions against *C. histolyticum* collagenase (ChC) and porcine pancreatic elastase (PPE) were measured spectrophotometrically according to the method used by Wittenauer et al., (10) by using a multi-mode Tecan GENios microplate reader equipped with analysis software Xfluor4 version 4.51 (Salzburg, Austria). Both enzymes were incubated with the extracts and their CGA fractions with relevant substrates (see below). The inhibitory potential of the grape pomace extracts were examined in dilutions so as to establish a dose-dependent curve in order to calculate the half-maximal inhibitory concentrations (IC_{50}). Due to the high concentration of polyphenols in the grape pomace extracts, the dilutions of 1:50 to 1:200 with total polyphenolic contents ranging from 52.5 to 7.8 mg GAE/L were applied before being incubated with collagenase and elastase.

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

a. Dilutions of ethanolic and hot water grape pomace extracts with water at concentration of (1:50), (1:100) and (1:200) (extract:water).

b. CGA and liquid fractions derived from CGA separations generated from TWEEN20 surfactant.

c. Aqueous solution of gallic acid (43 mg/L, 85 mg/L, 128 mg/L and 170 mg/L).

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

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

Elastase (PPE) assay: porcine pancreatic elastase (PPE) inhibitory activity of the individual samples (a-c) was determined spectrophotometrically by using the AAAPVN as the substrate and by monitoring the production of p-nitroaniline at 405nm to determine the reaction rate. Briefly, 10 µl was taken and loaded into wells together with 100 µl of Tris buffer and 30 µl of samples. The mixture was incubated for 20mins at 25°C. Subsequently, 40 µl of the AAAPVN (dissolved in 2mM Tris buffer at 0.25mg/mL) was added. Since the PPE was performed with AAAPVN as the substrate peptide, the enzyme activity can be calculated from the released of p-nitroaniline as a product, leading to the increased in absorption values. The absorbance was monitored for 20mins after the addition of AAAPVN and the initial velocities, the inhibitory effect and IC_{50}. The values were calculated as in Eq.2.

Statistical analysis

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

RESULTS AND DISCUSSIONS

Ethanolic and hot water extraction

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

Polyphenol composition of crude grape pomace extracts

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

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

Concentration (mg/L)						
Compound/Sample	**EE**	**CGA**	**LP**	**HWE**	**CGA**	**LP**
Phenolic acids						
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
Flavonols						
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
		Flavanols				
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
Anthocyanins						
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

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

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

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	* *trans*-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	√	√

All compounds were confirmed according to Kammerrer et al. (22).
*compounds were confirmed with pure standards.
EE: ethanol extract; HWE: hot water extract.

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

In general, both extracts had the same type of compounds present but interestingly they differed in their composition. This is particularly clear when the mass percentage of groups of polyphenols (eg: phenolic acids) is calculated from data in Table 1. For example, phenolic acids were present at higher proportion in the HWE (37%) than in the EE (17%); in both extracts gallic acid was the predominant phenolic acid. Similarly flavanols where at higher proportion in HWE (14%) than in EE (7%). However the composition of flavonols was similar in both extracts, (34% and 27% in EE and HWE respectively) but quercetin was predominant in EE and Keampferol in HWE. The anthocyanins composition was higher in EE (43%), than in HWE (22%). However, with regards to the collagenase and elastase inhibitory activities and their relationship to polyphenols composition (see below) phenolic acids, flavonols and flavanols were the most relevant as anthocyanins have not been related to these activities.

Separation of polyphenols from crude grape extracts by CGA

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

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

Extract	**EE**	**HWE**
V_{CGA}/V_{feed}	16	16
$V_{LP}/V_{CGA\text{-}phase}$	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}

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

Collagenase and elastase inhibitory activity

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

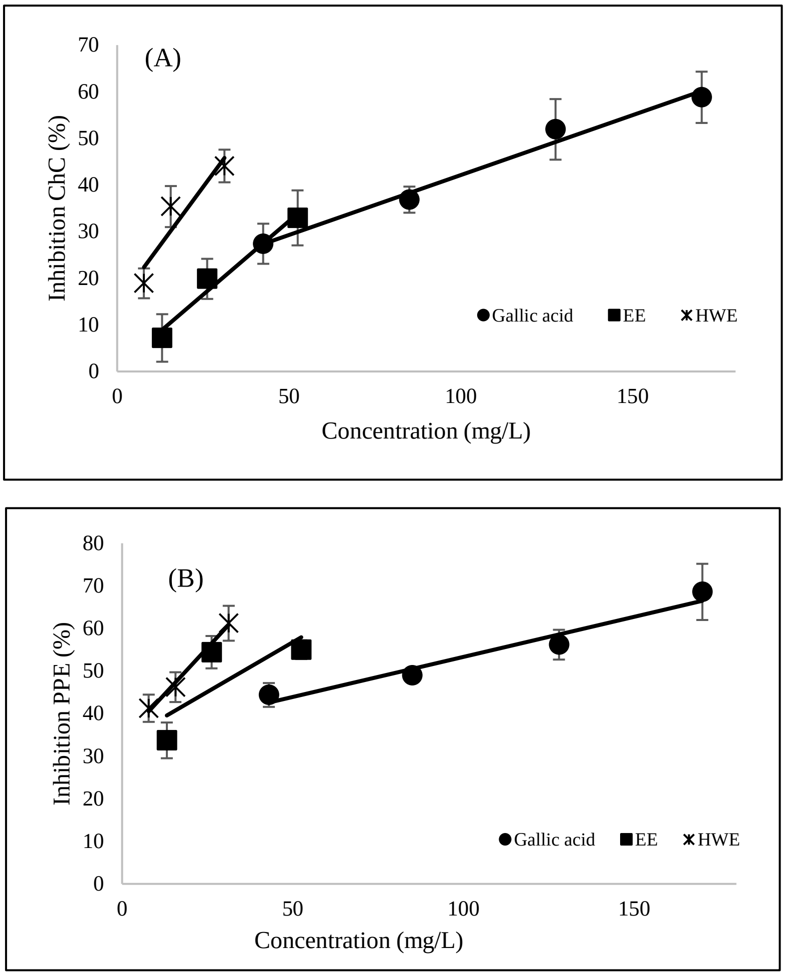

Figure 2: Dose dependent inhibition of collagenase (A) and elastase (B) activity by gallic acid, ethanol extract (EE) and hot water extract (HWE) (n =3).

The same trend was observed for PPE inhibitory activity. Elastin is an insoluble fibrous protein which occupies only 2-4% of the skin dermis weight but plays a vital role ensuring the elasticity of the skin (6). Based on the IC_{50} results, HWE (18.7mg/L) had the highest potency as compared to EE (35.5mg/L) and galic acid (82.0mg/L). Similar IC_{50} value was obtained with the methanolic extract of grape pomace (14.7mg/L) which may suggests comparable polyphenol composition (10).

The higher inhibitory activity of HWE than EE against collagenase and elastase can be explained by the differences in polyphenols composition. The phenolic acids such as gallic acid and chlorogenic acid in HWE which account for 37% of total polyphenols could have a pronounced effect on the inhibitory activities. Gallic acid, a low molecular weight hydrophilic compound could play an important part in the observed activity by accessing the active centre site of the elastase and blocking the binding of substrates to this site (10). However, given that the potency of the extract was superior than that of the gallic acid alone (Table 4), it is clear that other components also may contribute to the activity, perhaps in a synergistic manner. Chlorogenic acid, for example, which is a derivative of cinnamic acid, could also contribute as it is well known for its potent antioxidant and anti-inflammatory activities (25). Moreover, the catechin and epicatechin which were present at high proportion in HWE (14%) could interact with the elastase by hydrophobic interactions, causing conformational changes of elastase and thus increasing the inhibitory activity (7). On the other hand, EE had high composition of flavonols, particularly quercetin and resveratrol but they are larger molecules with lower solubility in water than the phenolic acids which could possibly limit their activity.

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

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

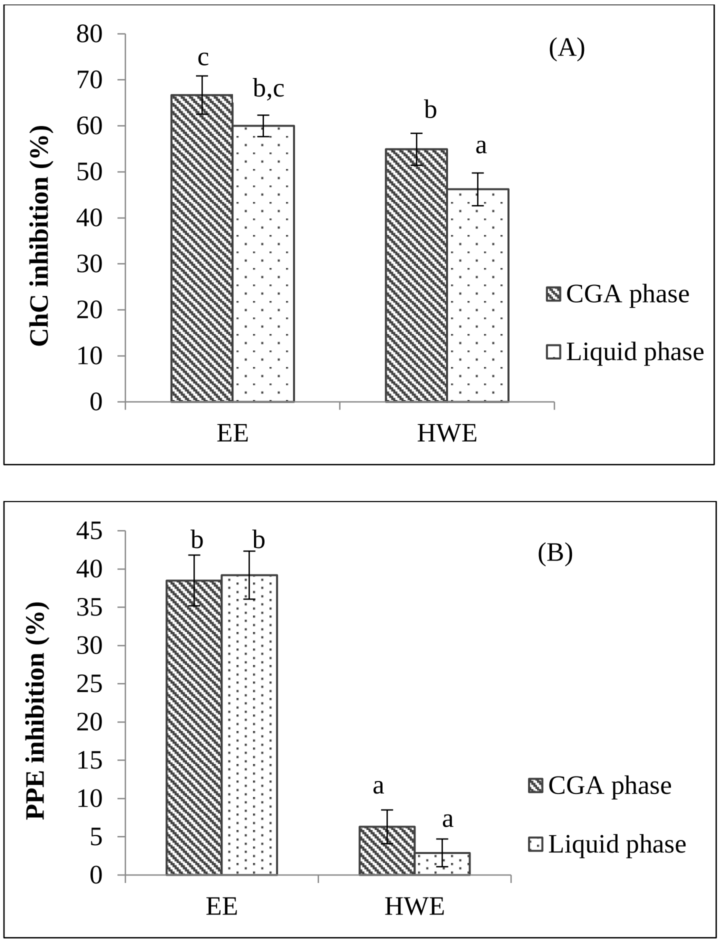

Figure 3: Anti-collagenase activity (A) and anti-elastase activity (B) of CGA fractions from EE and HWE. Bars are means ± standard deviation of three determinations (n = 3). Same superscript letters indicates means with no significant difference ($p>0.05$) according to ANOVA (n=3).

For example, the composition of phenolic acids in CGA-HWE and LP-HWE were almost the same (mass percentages of phenolic acids over total phenols were 47% and 45% respectively) and for flavonols composition was higher in the liquid phase (31% in CGA and 47% in liquid phase). The same trend was noted in CGA-EE and LP-EE where phenolic acids and flavonols compositon was very similar in both fractions (4.5 and 5.2% phenolic acids in CGA and LP respectively and 26% flavonols in both fractions). This similarity in compositon supports the insignificant differences in inhibitory activities of these fractions against both enzymes. Kaempferol was found at high concentration in both CGA-HWE and LP-HWE (23.5 and 24.4 mg/L respectively) but in the case of CGA-EE, the most predominant flavonol was quercetin (31.1mg/L). These compounds could possibly be the main contributors to the inhibitory activities observed whereby the hydroxyl group in C-3 might played a role in conferring the inhibitory activity (9). Moreover, the high content of gallic acid in CGA-HWE (24.9mg/L) and in LP-HWE (12.2mg/L) could also be important for the ChC inhibitory activity. The hydroxyl group from gallic acid could act as a hydrogen bond acceptor/donors with the hydroxyl, amino or carboxyl groups of the collagenase's side chain functional groups which can alter its structure, while the benzene rings of the polyphenols can form hydrophobic interactions with collagenase (8,10).

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

Table 4: Inhibitory efficiency (%/mg $GAE_{FI}L^{-1}$) 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

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

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

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

From results above it could be hypothesised that TWEEN20 might play a role in facilitating the delivery of the polyphenols to the target site of the collagenase. This explained why the efficieny of the CGA-EE increased susbstantially as compared to the crude extract's and it was comparable to that of CGA-HWE. It is also worth mentioning that the surfactant did not inhibit or activate both ChC and PPE (data not shown) hence, the inhibitory activities were solely due to the action of polyphenols in the fractions. Non-ionic surfactants were known to cause the least irritating effect to skin compared to anionic surfactants hence they were preferred for inclusion in many skin care products (28). Moreover, surfactants in general are known to alter the skin permeation by forming non-specific hydrophobic interactions involving the alkyl chains of the surfactant and the hydrophobic regions of the keratin in stratum corneum (30). Most studies about non-ionic surfactants and biological activities revealed that the C12 alkyl chain was the most important character in terms of perturbation of the membrane which explained the surfactant solubility and partitioning (31). Although most studies revealed that their interactions with non-ionic surfactants did not alter skin permeation to a significant level, enhancement has been noted in some studies whereby penetration of lidocaine (a type of drug) significantly increased through hairless mouse skin with TWEEN20 and TWEEN60 (28).

CONCLUSIONS

The extraction of polyphenols from grape pomace by ethanol and hot water led to crude extracts with different polyphenol compositions and this also resulted in differences in collagenase and elastase inhibitory activity. Phenolic acids were present at higher proportion in the HWE (37%) than in the EE (17%) which suggested their important role in the inhibition. HWE was the most efficient at inhibiting both collagenase and elastase and both EE and HWE were superior to gallic acid. Further extraction by CGA led to higher inhibitory efficiency against collagenase although there was no difference in efficiency between the separated phases for EE but there was for HWE. Gallic acid was the main component of the CGA-HWE but other polyphenols (eg: kaempferol) must have contributed also to its potency as this fraction inhibited collagenase over six times more efficiently than gallic acid. The CGA-EE fraction was found to be about four times more efficient than its crude extract and over six times more efficient than gallic acid in collagenase's inhibition; quercetin was found to be the major polyphenol in this fraction. These results suggested that although quercetin was highly insoluble in water and had high molecular weight, TWEEN20 helped to improve its solubility and therefore facilitated its delivery to the enzyme. Therefore, CGA separation led to fractions enriched in active polyphenols with enhanced collagenase inhibitory activity in both CGA and liquid phases. Although the polyphenols composition in CGA and liquid phases in both extracts were very similar, and hence their inhibitory activities, it must be stressed that further separation with CGA led to CGA fractions with less sugar and protein (and ethanol when applied to the ethanolic extract) which can be an advantage in terms of formulation. It should be noted that the concentration of these polyphenols in the CGA fractions were topically relevant (generally between 25-100µM). Moreover the surfactant in these fractions could act as a carrier and solubilising agent to enhance the permeation of polyphenols across the skin. Therefore, the surfactant rich solution may provide an optimum media that could facilitate the permeation of the polyphenols through the skin. This research shows the potential of CGA to revalorise the grape marc and to obtain an extract with potential in cosmetics applications.

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