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Research Article

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Effect of solvent, pH, extraction time and temperature on the extraction of phenolic compounds and antioxidant activity of *Carpobrotus edulis*

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ABSTRACT

Two 2^3 factorial design studies were performed to determine the effect of solvent, pH, extraction time and temperature on the extraction of phenolics and radical scavenging activity contained in crude leaf extracts of *Carpobrotus edulis*. For the aqueous model, pH 9, 72 h and 40 °C provided the highest yield (31.03 ± 0.58%), while the highest yield for the methanolic extracts was obtained using the same temperature and pH, but with a longer extraction time of 168 h (64.21 ± 2.12%). Quantitative phytochemical analysis was performed to determine the amount of phenolics, tannins and flavonoids contained in the crude extracts. When water was used as an extraction solvent, the highest concentration of phenolics was obtained using pH 9 and extracting for 72 h at 40 °C (6.42 ± 0.03 milligrams Gallic Acid Equivalent per gram of extract). However, when methanol was used as a solvent, the highest concentration of phenolics was obtained when the same pH and time of extraction was used, but at a lower temperature (25 °C) – 7.44 ± 0.50 mg GAE/g of extract. Antioxidant activity was determined using the ABTS and DPPH assays. For both, methanol extracts produced lower IC_{50} values than the aqueous extracts. The best combination of extraction conditions for aqueous extracts is pH 9, and 72 h of extraction at 40 °C. This produced the lowest IC_{50} values for both assays (298.28 µg/mL for DPPH and 140.77 µg/mL for ABTS assay). When methanol is used as a solvent, the extraction conditions producing the lowest IC_{50} values were pH 9, 72 h and 25 °C for the DPPH assay (109.84 µg/mL), and pH 5, 168 h and 25 °C for the ABTS assay (26.79 µg/mL). These values are all higher than for the positive control, ascorbic acid. A positive correlation exists between phenolic content and radical scavenging activity – higher phenolic content resulted in higher radical scavenging activity. Pearson's correlation coefficient was higher for the aqueous extracts than for the methanol extracts. Thus, extraction conditions must be modified to maximise extraction of phenolics, to obtain maximum radical scavenging activity.

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INTRODUCTION

Highly reactive free radicals and reactive oxygen species (ROS) damage DNA, protein and lipids, and play a role in the initiation of various chronic and degenerative diseases such as coronary heart disease, inflammation, stroke and diabetes (Scalbert *et al.*, 2005). The generation of these radicals or reactive oxygen species (ROS) during metabolism gives rise to oxidative stress, which leads to disease. These harmful effects can be reduced by endogenous antioxidant enzymes and non-enzymatic antioxidants, but these systems cannot fully avert the effects of oxidative stress (Subedi *et al.*, 2014). Supplementation of the diet with antioxidants, natural or synthetic, may help to reduce the damage caused by free radicals and ROS.

Antioxidants are molecules that delay or inhibit oxidative damage to target molecules, lowering the risk of disease. Such molecules must be readily absorbed, quench free radicals and chelate metal ions (Rahman, 2007). Interest in natural antioxidants, such as those found in plants, has increased in recent years. Plant-derived antioxidants are being studied as a safer alternative to synthetic antioxidants, which have side effects (Subedi *et al.*, 2014).

Phytochemicals such as phenolic acids, polyphenols and flavonoids have been shown to possess antioxidant activity and have a potential role in the prevention of certain diseases (Hausladen & Stamer, 1999). These phenolic compounds have properties that allow them to act as reducing agents, hydrogen

donors, singlet oxygen quenchers and metal chelators. The antioxidant properties of phenolic compounds are thought to be due to the following mechanisms: scavenging radical species such as ROS and RNS (reactive nitrogen species), suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in radical production, and upregulating or protecting antioxidant defense (Cotelle, 2001).

C. edulis is a commonly used medicinal plant in South Africa (van Wyk *et al.*, 2009). In this study, the phytochemistry and antioxidant activity of *C. edulis* was studied. *Carpobrotus edulis* L. Bolus, from the family Aizoaceae, is a perennial, evergreen, succulent plant with smooth, triangular-shaped leaves and large yellow flowers. *C. edulis* forms part of the *Materia medica* of South Africa (van Wyk, 2008; De Beer & Van Wyk, 2011). Several studies have shown *C. edulis* to have antioxidant, immunomodulatory, antimicrobial, antitumor and anticholinesterase activity (van der Watt & Pretorius, 2001; Ordway *et al.*, 2003; Martins *et al.*, 2010; Falleh *et al.*, 2011; Custódio *et al.*, 2012; Omoruyi *et al.*, 2012, 2014).

Characterisation of compounds responsible for these properties depends on the effectiveness of their extraction from the raw material. Phytochemical screening of *C. edulis* extracts has been performed and the yield of phenolics, flavonoids, tannins, saponins, and other phytochemical compounds was found to be affected by the solvent used in extraction (Omoruyi *et al.*, 2012). Their study only varied the extraction solvent. In addition to the solvent, the method of extraction, extraction time and conditions all affect the efficiency of extraction with regard to the nature of the desired phytochemicals (Tiwari *et al.*, 2011). These factors have not been fully investigated about their influence on the bioavailability of phenolics and antioxidants from *C. edulis*.

The presence of phenolics and antioxidants in *C. edulis* indicates that it can be used as an immunomodulatory agent, as well as an aid in wound healing. Numerous studies have supported the use of *C. edulis* extracts in repairing wounds. Bazzicalupo *et al.* (2021) found that aqueous extracts increased wound closure rates, stimulated fibroblast collagen production and inhibited collagenase. This was attributed to the high phenolic and flavonoid content in their extracts. One of these phenolics, chlorogenic acid, was found to accelerate excision wound healing in rats. In addition, there was an improvement in cell proliferation, and increases in TGF-β1 and collagen IV (Chen *et al.*, 2013). The anti-inflammatory activity of this plant was demonstrated by its inhibition of 15-LOX (lipoxygenase) and COX (cyclooxygenase) (Mulaudzi *et al.*, 2019). Nitric acid production was also reduced in microglial cells stimulated with lipopolysaccharide (Rocha *et al.*, 2017).

This study explored the influence that the pH, extraction time and temperature have on the bioavailability of simple phenols, tannins and flavonoids in crude leaf extracts of *C. edulis*, as well as their antioxidant activity.

MATERIALS AND METHODS

Chemicals and Solvents

The following reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO): aluminum chloride, Folin-Ciocalteau, gallic acid, methanol, polyvinylpyrrolidone K90 and quercetin. Potassium acetate and sodium carbonate anhydrous were obtained from Merck (Germany).

Collection and Authentication of *Carpobrotus Edulis*

Healthy leaves of *C. edulis* were harvested in June 2017 from established plants on the sand dunes along the Bloubergstrand coast (Latitude: -33°47'49.92" S and Longitude: 18°27'43.20" E) in South Africa. Dried voucher specimens (numbers 14830 and 148301) were identified by Professor Stefan Siebert and deposited at the AP Goossens Herbarium (Northwest University, Potchefstroom). The leaves were washed in tap water, rinsed twice in deionised water and frozen at -20 °C. The frozen leaves were lyophilised in a Scanvac CoolSafe™ (LaboGene, Lynge, Denmark) at -57 °C for 3-4 days. The dried plant material was pulverised using a Pulverisette 14, FRITSCH (Idar-Oberstein, Germany) and stored at -20 °C in a dark airtight container until the extraction process.

Experimental Design and Extraction Process

Two experimental designs were used for the screening and optimisation of variables affecting the aqueous and methanolic extraction of phytochemicals from *C. edulis*. In both the factorial designs, three independent variables, pH, extraction time and temperature, each at two levels, were screened forming the 2^3 full factorial designs. The independent variables with assigned letters and their respective levels are presented in Table 1.

The solid/solvent ratio (1:20) was kept constant for all experimental runs. The experimental runs were randomised to minimise the effects of unexpected variability in the observed response. The extraction yield (%) was considered as the dependent variable or response.

The independent variables (pH, extraction time and temperature) for each experimental run, were chosen as dictated by the experimental design matrix (Tables 2 & 3). Extractions were carried out using a shaking incubator (Labotec, Model 355, Johannesburg, South Africa) at 150 rpm. The extracts were filtered through Whatman No. 1 filter paper and the filtrates were lyophilised (aqueous extracts) or concentrated under rotary

Table 1: Independent variables and their levels studied in the 2^3-factorial design

Independent Variables	Low level (-)	High level (+)
A=pH	5	9
B=Extraction time	72 h	168 h
C=Temperature	25°C	40°C

Table 2: Design and response values of the 2^3 full factorial design for the aqueous extraction

Run	A	B	C	A	B	C	Extraction yield (%) ± SD	
							Actual	Predicted
01	-	-	+	5	72 h	40°C	25.66 ± 1.22	25.57 ± 0.99
02	+	-	+	9	72 h	40°C	31.03 ± 0.58	31.11 ± 0.99
03	-	+	-	5	168 h	25°C	24.43 ± 0.85	24.34 ± 0.99
04	+	+	-	9	168 h	25°C	22.21 ± 1.08	22.29 ± 0.99
05	-	+	+	5	168 h	40°C	24.54 ± 1.57	24.45 ± 0.99
06	+	+	+	9	168 h	40°C	21.55 ± 1.12	21.64 ± 0.99
07	-	-	-	5	72 h	25°C	24.95 ± 0.77	24.86 ± 0.99
08	+	-	-	9	72 h	25°C	24.09 ± 0.42	24.18 ± 0.99

evaporation (methanol extracts) (HB 10 basic, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The concentrated methanolic extracts were further dried until a constant weight was achieved. The extracts were then stored at -20°C in dark containers until further analysis. The extraction yield (%) was expressed as shown in Equation 1:

$$\%\,Extraction\,yield = \frac{amount\,(g)\,of\,dried\,crude\,extract\,obtained}{amount\,(g)\,of\,finely\,ground\,plant\,material\,used} x100 \quad (1)$$

Factorial Regression Model and Statistical Analysis

All experimental runs were carried out in triplicate to obtain a reliable estimate of the experimental error and to reduce the noise and bias for the observed response values. The response values were analysed using Design Expert version 11 (Stat-Ease Inc., MN) statistical software and fitted to a regression model (with three variables and their interactions) to describe the behaviour of the various independent variables and to predict the output and its successful optimisation.

Quantitative Phytochemical Analysis

Determination of the total phenolic content

The total phenolic content (TPC) in the various *C. edulis* extracts was determined using the Folin-Ciocalteau colorimetric method as described by Ainsworth and Gillespie (2007) with slight modification. This assay is based on the reduction of phosphomolybdic/phosphotungstic acid complexes by phenolic compounds in the plant extract. The intensity of the blue-colored complexes that resulted was determined spectroscopically (765 nm). Briefly, 200 µL of each crude extract (10 mg/mL) and 400 µL Folin-Ciocalteau (10% v/v) reagent were added into tubes and vortexed for 15 seconds. To each mixture, 1.6 mL of a sodium carbonate solution (700 mM, Na_2CO_3) was added and incubated at room temperature for 2 h for color development. At the end of the incubation period, the 200 µL reaction mixture was transferred into a 96-well microtiter plate and the absorbance was determined at 765 nm using an EPOCH 2 microplate reader (BioTek, Vermont). Methanol was used as the negative control for the experiments. The total phenolic content in the samples was calculated as milligram gallic acid equivalents (GAE) using the regression equation (y = 3.7695x + 0.0445) obtained from the standard curve where y is absorbance and x is mg/mL gallic acid. The total phenolic contents of the extracts were calculated using Equation 2. The experiment was conducted in triplicate and the results were expressed as mean ± SD values.

$$TPC = \frac{GAE\,(mg/mL)\,x\,Volume\,of\,extract\ \ (mL)}{weight\,of\,dried\,sample\,(g)} \quad (2)$$

In Equation 2, GAE (mg/mL) represents the gallic acid equivalent concentration from the calibration curve.

Determination of the total tannin content

The total tannin content (TTC) present in the crude extracts was calculated as the difference between the total phenolic content before and after the precipitation of tannins using the polyvinylpyrrolidone (PVP K90, Sigma-Aldrich) binding method as described by the Spectronomics protocol (Spectranomics, 2011). A mixture containing 500 µL dissolved PVP (10 mg in 500 µL of methanol) and 500 µL of the crude extract was vortexed and incubated on ice for 30 minutes. Following incubation, the mixture was centrifuged for 2 minutes at 10 000 x *g* (Heraeus Pico17, Germany). The tannin precipitation was repeated by transferring 750 µL supernatant into a centrifuge tube containing 10 mg PVP. The mixture was vortexed and incubated on ice for 30 minutes. After centrifugation, 200 µL of the supernatant was removed and the total phenolic content of the non-precipitated phenols determined using the Folin-Ciocalteau colorimetric method as described above.

Total flavonoid content

The total flavonoid content (TFC) present in each extract was determined using the Aluminium Chloride colorimetric method as described by Chang *et al.* (2002) with slight modifications. This assay is based on the formation of acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxy group of flavones and flavonols as well as the orthodihydroxyl groups in the A- or B-ring of flavonoids (Kalita *et al.*, 2013). In brief, a mixture containing 500 µL of crude extract (10 mg/mL), 100 µL of a 20% (w/v) aqueous aluminum chloride ($AlCl_3$) solution, 100 µL of a 1 M potassium acetate (CH_3COOK) solution, 1.5 mL methanol and 2.8 mL ultrapure water was incubated for 30 min at room temperature. After incubation, 200 µL of the reaction mixture was transferred into a 96-well microtiter plate and the absorbance was measured at 415 nm using an EPOCH 2 microplate reader (BioTek, Vermont). Distilled water (aqueous extracts) and methanol (methanolic extracts) were used as negative controls. The TFC in each sample was compared to the quercetin standard curve (y = 1.8355x + 0.0098) and expressed as milligrams of quercetin equivalent (QE) per gram of sample (mg QE/g). The experiment was conducted in triplicate and the results were expressed as mean ± SD values.

Antioxidant Activity

DPPH radical scavenging assay

The ability of the extracts to scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl) was measured using the microplate method described by Prieto (2012). A 0.2 mM solution of DPPH was prepared by dissolving 39.4 mg in 1 mL methanol. After vortexing, the solution was made up to 250 mL with methanol. The solution was stored at 4ºC in the dark until use. All extracts (aqueous and methanolic) were then loaded into the wells of a 96-well plate. Each extract was double-diluted with water so that the first well contained 500 µg/mL and the eighth well 3.906 µg/mL. A 2 mg/mL stock solution of ascorbic acid was also loaded and diluted to give the same concentrations – this was used as the positive control. Ultrapure water was used as a blank. A 100 µL of DPPH was then added to each well (containing diluted sample, diluted ascorbic acid, or water) and the plate was incubated at room temperature in the dark for 30 minutes. The absorbance was then read at 517 nm using an EPOCH 2 microplate reader (BioTek, Vermont). Radical scavenging activity was calculated using the formula:

$$\%inhibition = \frac{Abs(blank) - Abs(test\,sample)}{Abs(blank)} \times 100 \quad (3)$$

Samples were measured in triplicate and IC_{50} was calculated using linear regression analysis. Pearson's correlation coefficients were then calculated to determine any relationship between DPPH scavenging activity and phenolic concentration. All calculations were performed using Microsoft Excel.

ABTS Radical Scavenging Assay

The method used here is based on the original method developed by Re *et al.* (1999) and modified by Lee *et al.* (2015) for use with 96-well plates. Five millilitres of a 7 mM solution of ABTS was mixed with 88 µL of potassium persulphate and incubated in the dark at room temperature overnight to allow for free radical generation. After approximately 16 hours, the solution was diluted with water to an absorbance of approximately 0.75 at 734 nm. All tests were conducted in triplicate. Filtered samples were loaded in the wells of the plate and were double-diluted from 500 µg/mL to 3.906 µg/mL. Ascorbic acid at the same concentrations was used as a control. Ultrapure water was used as a blank. To each well containing 100 µL of sample/ascorbic acid or water only, 100 µL of ABTS was added. The plate was incubated at room temperature for 6 minutes and the absorbance was read at 734 nm using an EPOCH 2 microplate reader (BioTek, Vermont). Radical scavenging activity was calculated using the same formula as for DPPH (Equation 3) and IC_{50} values were determined using linear regression in Microsoft Excel. Pearson's correlations were calculated to determine the relationship between ABTS scavenging activity and the presence of phenolics.

RESULTS AND DISCUSSION

During the extraction process, a solid to solvent ratio of 1:20 was maintained. Thus, all reactions contained 10 g of powdered leaf material and 200 mL solvent. Each run was performed in triplicate.

The yields listed in Table 2 were obtained using water as a solvent and varying pH (A), extraction time (B) and temperature (C). The yields obtained using methanol and the same variables are listed in Table 3.

Quantitative numerical optimisation of the aqueous and methanolic extraction conditions was used to optimise the regression models for maximum extraction yield. For the aqueous model, a pH of 9, extraction time of 72 hours and temperature of 40 °C was calculated to deliver an optimal yield (31.03 ± 0.58%). When water is used as an extraction solvent, extraction time and temperature are significant factors ($p < 0.05$). For the methanolic model, a pH of 9, extraction time of 168 hours and temperature of 40 °C was calculated as having the potential to deliver an optimal yield of crude extract (64.21 ± 2.12%). When methanol was used as a solvent, extraction temperature was found to be a significant factor ($p < 0.0001$). Similar results for the extraction yields have been observed by another member of the Cell Biology Research Group and reported in their thesis (Terblanche, 2020). The same extraction conditions were used.

Qualitative determination of phytochemicals in the *C. edulis* extracts is also contained in the thesis mentioned above. Reducing sugars, phenols, tannins, flavonoids, diterpenes, cardiac glycosides, triterpenoids and phytosteroids were detected in all aqueous and methanolic extracts. Furthermore, the protein was detected in all methanolic extracts and some aqueous extracts. Quantitative determination of total phenolics, tannins and flavonoids are listed in Table 4 (aqueous extracts) and Table 5 (methanolic extracts).

The results suggest that the extraction conditions have a significant effect on the yield of the specific phytochemical (Tables 4 & 5). For example, when water is used as a solvent (Table 4), it is important to perform the extraction at pH 9, with an extraction time of 72 h and at 40 °C (Run 2) to get the highest total phenolic content (6.42 ± 0.03 mg GAE/g of

Table 3: Design and response values of the 2^3 full factorial design for the methanolic extraction

Run	A	B	C	A	B	C	Extraction yield (%) ± SD	
							Actual	Predicted
01	-	-	+	5	72 h	40°C	52.45 ± 3.06	52.93 ± 1.94
02	+	-	+	9	72 h	40°C	64.05 ± 0.85	63.13 ± 1.94
03	-	+	-	5	168 h	25°C	43.54 ± 0.80	42.62 ± 1.94
04	+	+	-	9	168 h	25°C	52.35 ± 1.26	52.83 ± 1.94
05	-	+	+	5	168 h	40°C	58.51 ± 3.55	59.09 ± 1.94
06	+	+	+	9	168 h	40°C	64.21 ± 2.12	64.06 ± 1.94
07	-	-	-	5	72 h	25°C	41.85 ± 1.18	41.70 ± 1.94
08	+	-	-	9	72 h	25°C	46.08 ± 0.82	46.67 ± 1.94

Table 4: Quantitative phytochemical analysis of the crude aqueous extracts (± SD)

Run	Total phenolic content (mg GAE/g)	Total tannin content (mg GAE/g)	Total flavonoid content (mg QE/g)
01	6.28 ± 0.03	5.25 ± 0.12	0.17 ± 0.02
02	6.42 ± 0.03	2.94 ± 0.13	2.18 ± 0.10
03	3.55 ± 0.39	1.45 ± 0.47	BDL
04	4.12 ± 0.22	2.75 ± 0.27	2.91 ± 0.21
05	6.10 ± 0.64	3.98 ± 0.73	1.51 ± 0.01
06	6.15 ± 0.13	3.98 ± 0.22	2.35 ± 0.34
07	6.15 ± 0.65	2.75 ± 0.57	3.48 ± 0.07
08	5.75 ± 0.24	3.69 ± 0.21	4.43 ± 0.42

BDL=below the detection limit

Table 5: Quantitative phytochemical analysis of the crude methanolic extracts (± SD)

Run	Total phenolic content (mg GAE/g)	Total tannin content (mg GAE/g)	Total flavonoid content (mg QE/g)
01	6.72 ± 0.39	2.31 ± 0.00	2.76 ± 0.00
02	6.95 ± 0.10	2.83 ± 0.15	2.03 ± 0.66
03	7.19 ± 0.35	3.06 ± 0.37	3.04 ± 0.06
04	6.91 ± 0.46	2.69 ± 0.51	3.69 ± 0.17
05	6.36 ± 0.08	2.85 ± 0.20	2.80 ± 0.33
06	6.74 ± 0.77	2.47 ± 0.28	2.31 ± 0.86
07	6.94 ± 0.18	3.09 ± 0.17	2.29 ± 0.56
08	7.44 ± 0.50	2.76 ± 0.41	2.76 ± 0.07

extract). However, when methanol is used as a solvent, different conditions (pH 9, 72 h and 25 °C – Run 8 in Table 5) are needed to maximize the extraction of total phenolics (7.44 ± 0.50 mg GAE/g) from *C. edulis*.

In some situations, it may be desirable to maximize the extraction of several types of phytochemicals (phenolics, tannins and flavonoids). In such cases, if methanol is used as a solvent, then the extraction would need to take place for 168 h and at a temperature of 25°C. The pH is not important, and in this case, pH 5 or pH 9 can be used. However, when water is used as a solvent, the best yields of all three phytochemicals can be obtained when the extraction is performed at pH 9, for 72 h at 25 °C.

Analysis of the DPPH scavenging ability using Design Expert shows that from the variables studied (pH, extraction time, temperature), all except the extraction time are significant factors ($p < 0.0001$) when water is used as a solvent (Table 6). The best combination of variables which provides the lowest IC_{50} value for the aqueous extracts (298.28 μg/mL) corresponds with Run 2 (pH 9 and 72 h of extraction at 40 °C) (Table 6). The best combination of variables that yields the lowest IC_{50} value for the methanol extracts (109.84 μg/mL) is Run 8 (pH 9 and 72 h of extraction at 25 °C) (Table 7). The concentration of the extract is the only significant factor when methanol is used as a solvent ($p < 0.0001$).

Overall, the methanol extracts provide greater scavenging of DPPH (and therefore, lower IC_{50} values). The yields from aqueous extracts are lower, and this might account for the lower concentrations of antioxidants (and higher IC_{50} values) found in these extracts. The IC_{50} values for the extracts are considerably

Table 6: IC_{50} values (μg/mL) for crude aqueous extracts using DPPH and ABTS assays

Run	DPPH assay	ABTS assay
01	429.72	235.16
02	298.28	140.77
03	5144.89	508.99
04	6425.01	547.52
05	1249.91	331.71
06	1142.85	348.35
07	1304.63	302.56
08	1312.94	250.51
AA*	11.06	4.15

AA* is ascorbic acid, the positive control

Table 7: IC_{50} values (μg/mL) for crude methanolic extracts using DPPH and ABTS assays

Run	DPPH assay	ABTS assay
01	215.54	34.23
02	156.81	38.36
03	141.18	26.79
04	152.02	33.28
05	351.78	30.91
06	376.53	39.45
07	305.21	34.57
08	109.84	30.39
AA*	11.06	4.15

AA* is ascorbic acid, the positive control

higher than the value obtained for the positive control, ascorbic acid, which produces a value of 11.06 μg/mL. When Rocha *et al.* (2017) used the Soxhlet apparatus to produce extracts, the methanol extracts also exhibited the highest scavenging activity against the DPPH radical when compared with other organic solvents.

For scavenging of the ABTS radical, Design Expert's ANOVA function indicates that all extraction variables (pH, extraction time and temperature) are significant factors when water is used as a solvent ($p < 0.0001$) (Table 6). The combination of variables which provides the lowest IC_{50} value for aqueous extracts (140.77 μg/mL) is for Run 2 (pH 9, for 72 h at 40 °C). For methanol extracts (Table 7), the combination of extraction conditions that provides the lowest IC_{50} value (26.79 μg/mL) is pH 5, at 25 °C and 168 h (Run 3). Again, when methanol is used as an extraction solvent, only the concentration of the extract used in testing for radical scavenging ability is significant ($p < 0.0001$) As with the DPPH assay, extraction with methanol provides better results than with water (i.e. lower IC_{50} values). The IC_{50} values obtained for the extract in the ABTS assay are still considerably higher than the value obtained for the positive control, ascorbic acid, which is 4.15 μg/mL.

To determine the relationship between yield and phenolic content (where most of the antioxidant activity is contained), and the subsequent relationship between phenolic content and radical scavenging ability, Pearson's correlation tests were done in Microsoft Excel. For the aqueous extracts, there is a positive correlation between the yield of crude extract and total phenolic content ($r = 0.38$), but a slightly negative correlation for total

tannins (r = - 0.05) and total flavonoid content (r = - 0.15). For the methanol extracts, a negative correlation was calculated for each of the three types of phytochemicals. This finding suggests that an increase in extraction yield does not necessarily result in an increase in the availability of any of the phytochemicals under investigation.

There does appear to be a correlation between Total Phenolic Concentration (TPC) and radical scavenging activity. For these calculations, IC_{50} values were used. For the aqueous extracts, higher TPC values resulted in higher scavenging activity for both DPPH and ABTS (r = - 0.952 and r = - 0.879, respectively). For methanol extracts, a similar result was obtained (r = - 0.759 for DPPH and - 0.320 for ABTS). The negative correlation values are expected – as the concentration of phenolics increases, the IC_{50} decreases. There was a greater correlation between phenolic content and radical scavenging activity for the aqueous extracts than for the methanol extracts. The lower r values for the methanol extracts (even though they produced the lowest IC_{50} values) may suggest that other antioxidant molecules (in addition to phenolics), which were extracted by the methanol, are responsible for the scavenging of the two radicals.

Indeed, the Pearson coefficient (r) for Total Flavonoid Concentration (TFC) in the methanol extracts and radical scavenging activity was - 0.588 for ABTS and - 0.376 for DPPH. Coefficients for the aqueous extracts were closer to zero – there was little to no correlation between TFC and radical scavenging activity. There is a stronger correlation between total tannin concentration (TTC) in the aqueous extracts and scavenging activity (r = - 0.644 for DPPH and - 0.524 for ABTS). For the methanol extracts, the correlation between TTC and radical scavenging activity was r = - 0.136 for DPPH and – 0.458 for ABTS. Studies of other halophytes by Trabelsi *et al*. (2012, 2013) also showed a strong correlation between phenolic content and radical scavenging activity. On the other hand, a study by Conforti *et al.* (2008) showed there was no relationship between antioxidant activity and TPC in ethanolic extracts of selected Mediterranean dietary plants. They suggest that the type of phenolics determines radical scavenging activity, not their amounts.

For the extracts used in this study, the maximum yield of the crude extract is obtained when a higher temperature and pH are used, with methanol delivering a higher yield compared with water. When phytochemicals are extracted, lower temperatures are preferred, but the time of extraction depends on the solvent used. When using methanol as a solvent, a longer time of extraction results in greater phytochemical extraction. Methanol extracts provide greater radical scavenging activity than aqueous extracts. This may be due to the polarity of methanol and its ability to extract polar compounds such as phenolics and flavonoids, which have antioxidant properties (Alam *et al*., 2012).

The phenolic and flavonoid content of *C. edulis* supports evidence that this plant has wound-healing and immunomodulatory properties. Flavonoids have been found to accelerate wound healing by regulating collagen formation and decreasing wound closure time. Phenolic compounds, such as chlorogenic acid, stimulate the production of cytokines which are involved in tissue repair and regeneration. These include tumor necrosis factor (TNF-α) and transforming growth factor (TGF-β1), both of which are produced by macrophages (Chen *et al*., 2013; Bazzicalupo *et al*., 2021).

The effects of the extracts on the viability of cells would be the next step in determining whether this plant can be used in immune support and tissue repair for wounds. Furthermore, the effect of the extracts on cytokine and chemokine expression by immune cells such as macrophages would reveal the effects of the plant on the immune system.

CONCLUSION

When using the maceration technique, extraction conditions have a direct effect on the yields of crude extracts obtained from the leaves of *C. edulis*. In comparison with water, extraction with methanol resulted in higher yields, higher total phenolic concentration, and therefore, a higher radical scavenging activity against DPPH and ABTS.

Thus, *C. edulis* leaves can be used as a source of antioxidants, and these can be extracted using relatively simple methods. Extraction conditions should be modified to maximize the extraction of phenolics. This will result in higher radical scavenging activity. The presence of antioxidants and phenolics supports the use of *C. edulis* as a product for the treatment of inflammation and wound healing.

Thus, further research would involve determining which cytokines and chemokines (and receptors) are upregulated or downregulated by macrophages in response to extracts from *C. edulis*. This would reveal the effect *C. edulis* has on the processes of inflammation and tissue repair.

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