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Article

Chick pea water and chick pea polyphenols induce apoptosis and alleviate cell migration *in vitro* in human colon adenocarcinoma cells

Heidi Bochenek[ab], Nidhish Francis[c], Abishek B. Santhakumar[ab], Christopher L. Blanchard[ab] and Kenneth A. Chinkwo[ab*]

[1] *School of Dentistry and Medical Sciences, Charles Sturt University, Wagga Wagga, 2650, New South Wales, Australia*
[2] *Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, 2650, New South Wales, Australia*
[3] *School of Animal and Veterinary Sciences Charles Sturt University, Wagga Wagga, 2650, New South Wales, Australia*
* Correspondence: kchinkwo@csu.edu.au

Abstract: Chickpea is an essential legume, a staple food in many cultures and contains nutrients with potential health benefits. The chickpea water (CPW) leached out after cooking is usually discarded, which may potentially have significant anti-cancer and other health beneficial properties. This study compared the in-vitro bioactivity of CPW with chickpea polyphenol extract (CPPE) to evaluate its impact on pathways of colorectal cancer progression and development. Morphological observation by APOPercentage, cell viability detection using a cytotoxic assay and cell migration-scratch assay points to measure rate of metastasis were employed. Overall antioxidant activity of CPW and CPPE were measured using ABTS and DPPH free-radical assays. At 50 μg/mL concentration and above, both CPW and CPPE extracts significantly reduce cell viability in HT-29 colon cancer cell lines ($p < 0.05$). Moreover, a quantitative analysis of the extent of apoptosis demonstrated that at 250 and 500 μg/mL concentrations, both extracts induced significant apoptosis compared to the untreated control. Meanwhile, the cell migration scratch area decreases by 34.42% and 15.27% when treated with CPW and CPPE, respectively. In summary, CPW demonstrated comparable in vitro anti-cancer properties and antioxidant activity in colorectal cancer cells to CPPE. Further, in vivo studies are warranted to evaluate the physiological bioactivity of CPW and CPPE in targeting pathways of cancer development and progression.

Keywords: chick pea, chick pea water, apoptosis; cytotoxicity; colorectal cancer; polyphenols

1. Introduction

Chickpea (*Cicer arietinum* L.), is an important legume crop grown and consumed worldwide. Chickpea (CP) is composed of proteins, carbohydrates, fibre and certain vitamins, minerals, and polyphenols. The two most common CP varieties include Desi and Kabuli. Evidence suggests that CP consumption may have health beneficial properties by targeting specific mechanistic pathways of chronic conditions such as cancer and metabolic diseases (Anwar, Hussain & Mustafa, 2018; Chickpeas (Giusti, Capuano, Sagratini, & Pellegrini, 2019). More specifically, CP polyphenols and other bioactive compounds (such as butyrate, lycopene and biochanin A) have been demonstrated to have an impact on pathways of cancer pathogenesis (Jukanti, Gaur, Gowda, & Chibbar, 2012). For example, a major sterol present in CP, β-sitosterol has been shown to significantly reduce chemical-induced colon tumour in a rat model (Raicht, Cohen, Fazzini, Sarwal, & Takahashi, 1980). Nevertheless, by-products of CP processing such as CP water, hull and its associated cancer-preventive properties have been under investigated. Processing by boiling is

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the most common cooking method, which may impact CP's chemical composition, digestibility and bioavailability (Alajaji & El-Adawy, 2006). In most processing conditions, the broth or aqua-faba, commonly known as the chickpea water (CPW), is usually discarded after boiling. There is reported evidence that the discarded aqua-faba may contain bioactive polyphenols. It has also been demonstrated that chickpea polyphenols during the boiling process may leach out into the water consequently degrading to smaller more bioactive compounds (Giusti et al., 2019). For instance, in CP aqua-faba, lignin is broken down to syringic acid, which is known to contain significant anti-cancer properties (Giusti et al., 2019). Hence, it is key to profile and assess the bioactive properties of CP by-product polyphenols to explore their applications as a functional food alternative.

In the past, researchers have postulated several mechanisms through which dietary polyphenols target specific mechanistic pathways of cancer progression or development (Patra et al., 2021). The usual mode of action is by modulating molecular events and signalling associated with cell proliferation, survival, differentiation, migration, angiogenesis and immune responses. The antioxidant and anti-inflmmatory properties of polyphenols have been shown to be directly linked to this activity(Zhou et al., 2016). However, the most common mechanism is by resisting cell death by apoptosis (Hanahan & Weinberg, 2011). Cereal polyphenol extracts have been shown to activate pro-apoptotic genes, p53 protein, to induce cell signalling via caspases; and reducing metastasis initiated by MMPs (Rao, Chinkwo, Santhakumar, Johnson, & Blanchard, 2019).

The objective of this investigation was to evaluate the impact of CP and CPW polyphenols on pathways of cancer such as overall cell viability, apoptosis, cellular migration, and metastasis using an *in vitro* colorectal cancer (CRC) cell model.

2. Materials and Methods

2.1. Samples and chemicals

Pre-milled chickpea hull and whole desi chickpeas were provided by Woods Foods (Queensland, Australia) and the Department of Primary Industries (Wagga Wagga, New South Wales, Australia) respectively.

Chemicals including acetic acid, acetone, hexane, acetonitrile, ethanol, potassium persulfate, sulfuric acid and methanol were sourced from Chem Supply Pty Ltd (Port Adelaide, South Australia, Australia). 2,2'-azino-bis (3ethylbenothiazoline-6-sulfonic acid) (ABTS), Caffeic acid, Catechin , 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferulic acid, Folin-Ciocalteu reagent, Gallic acid, 6-Hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid (TROLOX) Vanillic acid, were sourced from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Polyphenol extraction

Whole desi chickpeas were ground in a Perten Laboratory Mill 3,000 (Hagersten, Sweden) fitted with a 0.4 μM sieve. Extraction of phenolic compounds from flour was conducted according to our group's previously standardised method (Rao, Santhakumar, Chinkwo, & Blanchard, 2019). The phenolic extracts were stored at −20°C and reconstituted in 50% methanol for chemical analysis. The extraction and chemical analyses were performed in triplicates for all samples.

2.3. Preparation of chickpea water

Whole desi chickpeas were autoclaved in a 1:20 ratio with water using the TOMY sx-700 autoclave (LabGear Milton Queens Land Australia), at 121°C for 15 min. The chickpea water (CPW) samples were then concentrated using a rotary evaporator (Rotavapor R-210 BUCHI Labortechnik, Flawil, Switzerland), and freeze-dried (Christ-Alpha 2–4 LD Plus freeze drier, Biotech International, Germany). Samples were reconstituted, centrifuged, filtered, and stored at -20°C until further use.

2.4. Total phenolic content (TPC)

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The total phenolic content was analysed according to the method described by (Rao, Santhakumar, et al., 2019) with minor modifications. Briefly, 125 µL of the Folin–Ciocalteu reagent was added to 125 µL of diluted crude chickpea polyphenol extract, or chickpea water samples along with 500 µL of deionised water and vortexed. After 6 min of incubation, the mixture was neutralised with 1.5 mL of 7% sodium carbonate and 1 mL of deionised water was added. The mixture was incubated for 90 min in the dark, and the absorbance was measured at 725 nm on a microplate reader (BMG Labtech Fluostar Omega, Offenburg, Germany). The total phenolic content of the rice samples was expressed as mg of gallic acid equivalents (GAE/100 g).

2.5. ABTS antioxidant analysis

The antioxidant capacity of the chick pea samples were measured using the method described by Zhou, et al. (2014). A 150 µL aliquot of each reconstituted and diluted sample were combined with 2 mL ABTS working solution and vortexed. Samples were left to react for 10 minutes at room temperature, in the dark. All samples were analysed in triplicate. A Trolox standard curve was used to quantify the radical scavenging activity of samples. Data was expressed as mg/g Trolox equivalents (TE).

2.6. 2,2-. Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

Free radical scavenging activity was quantified using a DPPH assay adapted from (Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011). CPW and CPPE extracts resuspended in 50% methanol (100 µL) were vortexed with 2000 µL of a DPPH (2.49 mg in 100 mL of methanol) radical solution. The mixture was incubated at room temperature in the dark for 30 min. The absorbance of the mixture was read at 715 nm at room temperature using a microplate reader. DPPH free radical scavenging ability was quantified expressed as µmol 100 g−1 Trolox equivalents (TE).

2.7. Cell culture

Two different human colon adenocarcinoma cell lines (SW480 and HT29) were employed. SW480 colorectal cancer cells were grown in Dulbecco's modified eagle's medium (DMEM), containing 10% FBS and 1% penicillin-streptomycin. HT-29 cells were maintained in McCoys 5a media and supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were grown in a humidified CO_2 incubator at 37°C. SW480 cells with passage numbers 7-12 and HT-29 cells with passage numbers 2-7 were used in this study.

2.8. Cytotoxicity assay

Cells were seeded in triplicates in a 96 well plate at a density of 3×10^4 cells/well and incubated for 24 hours before commencement of the resazurin red cytotoxicity assay as described in our previous work (Callcott, Blanchard, Oli, & Santhakumar, 2018). Post phosphate buffered saline wash, chickpea polyphenolic extracts (CPPE) and chickpea water (CPW) extracts at concentrations 25, 50, 100, 250, 500 and 1000 µg/mL were applied to the cells for 4, 8, 12 and 24 hours. 40 µM of H_2O_2 was used as a positive control in addition to a media-only negative control. After incubation, wells were washed again with PBS and 100 µl of resazurin red working solution was added to each well incubated for 4 h at 37°C and 5 % CO_2. The absorbance was measured at 570 nm and 600 nm. The percentage of cell viability was calculated as outlined by Ataollahi, et al. (2015).

2.9. APOPercentage assay

A Biocolor APOPercentage kit (Belfast, UK) was used to determine if the cytotoxicity observed was due to apoptosis. SW480 and HT-29 cells were seeded as outlined previously. Both extracts were prepared at concentrations 100µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml. A positive control of 40mM of hydrogen peroxide was used. A media sample

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without extracts was used as a negative control. Cells were incubated for an 8-hour incubation period, however, 30 minutes prior, media supplemented with extracts and 5% v/v APOPercentage dye was then applied to cells. After incubation, reagents were aspirated, and cells were washed three times with PBS. A Nikon Eclipse Ti compound microscope (Tokyo, Japan), was used in addition to a Nikon intensilight C-HGFI illuminator, a Digital Sight DS-U3 camera controller, a DS-Fi2 camera and the NIS-Elements imaging software (Melville, New York, NY, USA), for morphological analysis. ImageJ was then used to analyse the microphotographs.

2.10. Cell migration scratch assay

HT-29 cells were seeded into 24 well plates at a concentration of 3×10^5 cells/well and incubated for 24 hours to attain adherence and confluency. A control of cells with media was used. CPW and CPPE test samples were applied to cells at a concentration of 250 µg/ml. Time lapse videos were produced using a Nikon eclipse ti-e confocal microscope (Tokyo, Japan), in addition to using a Nikon intensilight C-HGFI illuminator, a Digital Sight DS-U3 camera controller, a DS-Qi1 camera and the NIS-Elements imaging software (Melville, New York, NY, USA). Okolab H301-K-Frame with Okolab CO2 unit with active humidity controller (Ambridge, USA) set to 5 and at a temperature of 37.5 degrees was used in conjunction with the microscope set-up. Every minute, for 72 hours a photo was taken of the cells. Videos were then analysed using ImageJ software.

2.11. Statistical analysis

Statistical analysis was conducted using one-way and two-way analysis of variance (ANOVA), followed by posthoc Tukey's multiple comparisons test using GraphPad Prism 7 software (GraphPad Software Inc, California, CA, USA). The results are reported as mean ± standard deviation. Statistical significance was determined at a level of $p < 0.05$.

3. Results

3.1. Total phenolic content and antioxidant activity

As expected, the TPC of CPPE (48.84 mg/g GAE) was observed to be significantly higher ($p < 0.05$) than CPW (5.50 mg/g GAE) (Figure 1A). Furthermore, the DPPH free radical scavenging activity was significantly higher ($p < 0.05$) for CPPE (22.2 mg/g TE) when compared to CPW at (8.41 mg/g TE) (Figure 1B). Nevertheless, the ABTS activity for the CPW was marginally higher than the CPPE at 29.65 mg/g GAE and 25.06 mg/g GAE respectively (Figure 1 C).

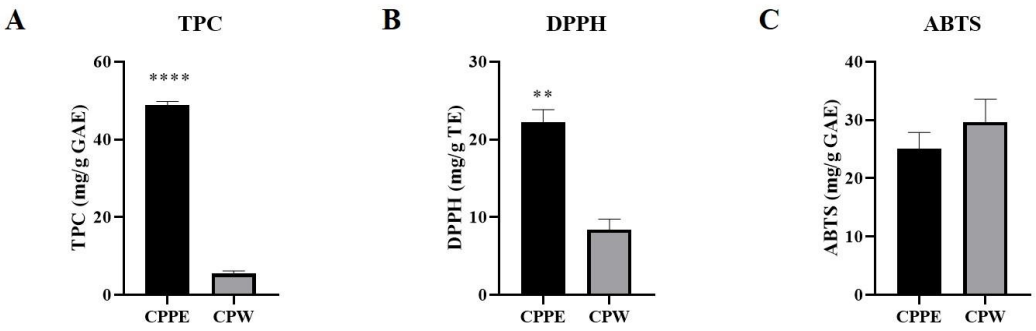

Figure 1: Total phenolic content (TPC) **(A)**, free radical scavenging (DPPH) **(B)** and antioxidant activity (ABTS) **(C)** of chickpea polyphenol extract (CPPE) and chickpea water (CPW). Data are presented as mean ± SD. Significance is indicated by asterisks, whereby *p <0.05, **p <0.01, ****p <0.0001.

3.2. Resazurin red cytotoxicity assay

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Both CRC cell lines (SW480 and HT29) were subjected to CPW and CPPE extracts for 8 hours to examine cell viability. Cells were treated with extracts at concentrations ranging from 25 µg/mL to 1000 µg/mL. A significant reduction in cell viability was observed across the two CRC cell lines post treatment with both CPW and CPPE. A non-dose-dependent decrease in cell viability was observed in SW480 cells post CPW treatment at concentrations of 100 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL ($p < 0.05$) (Figure 2A). Meanwhile, CPPE at concentrations of 250 µg/mL, 500 µg/mL, and 1000 µg/mL ($p < 0.05$) exerted a dose-dependent decrease in SW480 cell survival (Figure 2B). Both CPW and CPPE treatment at concentrations of 50 µg/mL and above on HT-29 cell lines significantly reduced cell viability ($p < 0.05$).

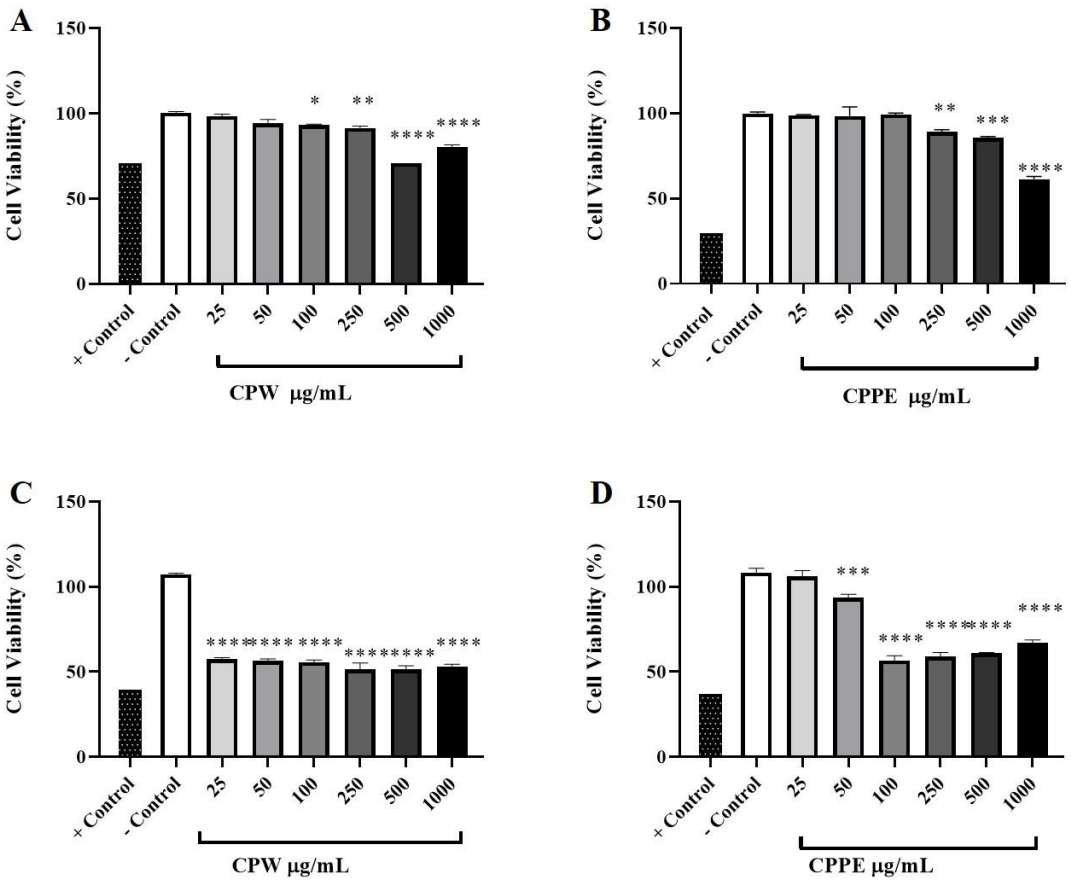

Figure 2. Effect of CPW and CPPE extracts on cell viability in SW-480 (A-B) and HT-29 (C-D) cells. Data are presented as mean ± SD. Significance is indicated by asterisks, whereby *p <0.05, **p <0.01, ****p <0.0001. CPPE- chick pea polyphenolic extract and CPW- chick pea water extract.

3.3. Effect of CPW and CPPE on apoptosis

The APO Percentage assay was used to assess the impact of CPW and CPPE on the extent of apoptosis on both SW-480 and HT-29 cells. At concentrations of 250 µg/mL and 500 µg/mL, CPW and CPPE exposed to SW480 cells, applied for 8 hours, stained pink and showed a morphological distortion of the cells, not seen in the untreated control (Figure 3A). A similar result was observed when CPW and CPPE at concentrations of 100 µg/mL, 250 µg/mL and 500 µg/mL were treated with HT-29 cells (Figure 3B). A quantitative analysis of the extent of apoptosis demonstrated that at concentrations of 250 and 500 µg/mL, both CPW and CPPE extracts induced significant apoptosis compared to the untreated control (Table 1).

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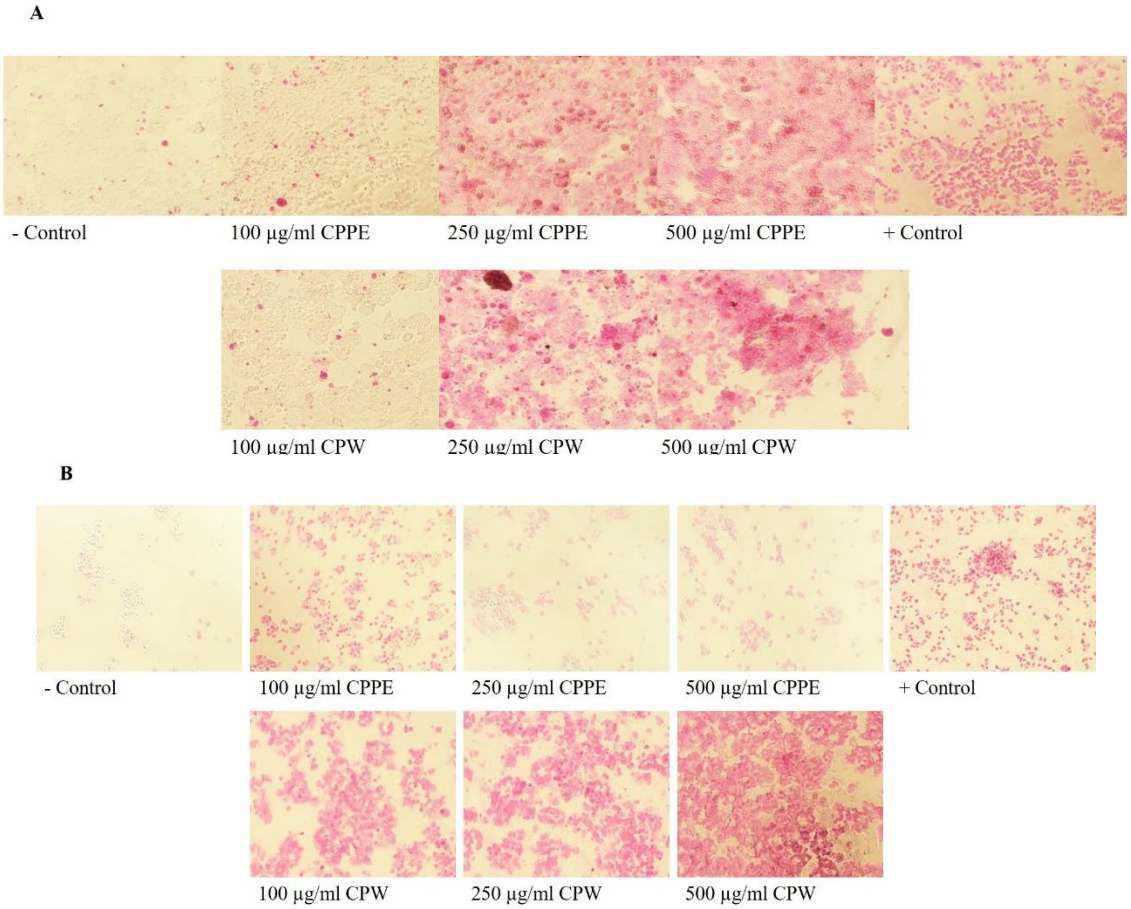

Figure 3. Effect of CPW and CPPE on apoptosis using APO Percentage assay. CPPE and CPW extracts at concentrations of 100, 250 and 500 µg/mL exhibits a pink colouration demonstrating apoptosis of SW-480 (A) and HT-29 cells (B). CPPE- chick pea polyphenolic extract and CPW- chick pea water extract.

Table 1: Extent of apoptosis exhibited by CPW and CPPE.

	SW-480		**HT-29**	
	CPW	**CPPE**	**CPW**	**CPPE**
0 µg/mL	32.31%	37.31%	3.17	5.66
250 µg/mL	*51.27%	*72.31%	*96.97	*63.14
500 µg/mL	*63.49%	*63.68%	*98.55	*95.33

3.4. Cell migration scratch assay

A cell migration experiment was employed to evaluate the effects of CPW and CPPE on cell metastasis in HT-29 cells. Photo-stills of time-lapse video were used to conduct the quantitative analysis of cell migration (Figure 4). Overall, both CPW and CPPE reduced the cell migration when compared to the control. Percentage of scratch closure showed that the most significant inhibition of cellular migration was observed post CPW treatment (250 µg/mL) when compared to CPPE and the control. The percentage of scratch

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area closure decreased by 34.42% and 15.27% when treated with CPW and CPPE respectively.

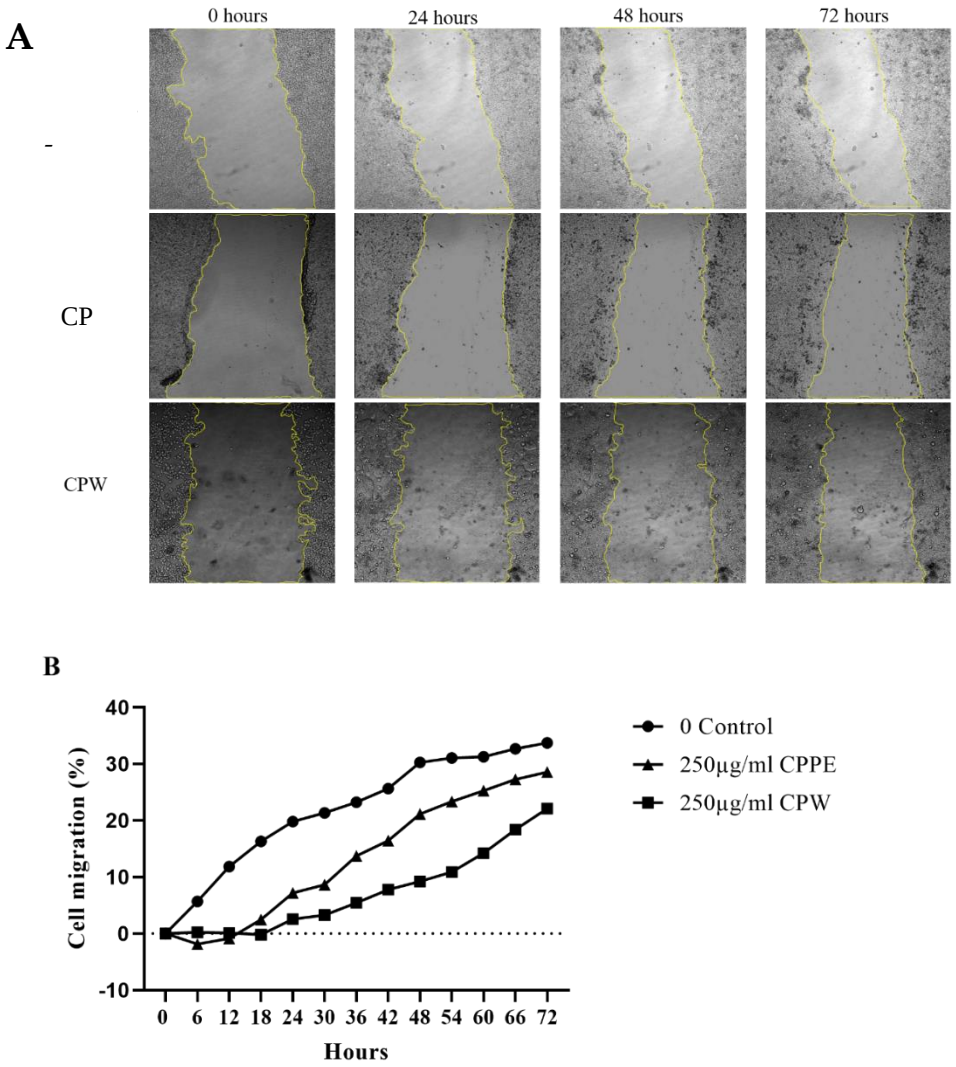

Figure 4. Cell migration assay of HT-29 cells exposed to 250 µg/mL of CPW and CPPE extracts. (A) Representative pictures of the area of scratch on HT-29 cells taken at the start of the experiment (0 hour), 24, 48 and 72 hours post treatment with the CPPE and CPW extracts. (B) Quantification of cell migration was undertaken by measuring the area of the scratch at 6-hour time points. Percentage of scratch closure showed that treatment with CPW extracts had a greater inhibition on cellular migration when compared to CPPE extracts.

4. Discussion

Chickpea (CP) contains bioactive compounds such as polyphenols that may impact the signalling pathways of cancer pathogenesis (Faridy, Stephanie, Gabriela, & Cristian, 2020). Processing of chickpeas by boiling, one of the most common methods for cooking CP, may leach these bioactive compounds from the CP into the water. This study aimed to characterise and explore the bioactive potential of polyphenols from CP and CPW on pathways of cancer using an *in vitro* model of colorectal cancer cells (SW480 and HT-29). Findings from this study demonstrate that CPPE and CPW regulates the viability, apoptosis, migration, and metastasis of cancer cells. A preliminary chemical analysis by TPC revealed polyphenols to be present in CPW, though significantly lower than CPPE (Figure 1). Antioxidant activity for CPPE and CPW extracts as measured by ABTS were found to be 25.06 and 29.65mg/g GAE, respectively highlighting the bioactivity of CPW and a comparatively similar antioxidant activity to the CPPE.

 doi:10.20944/preprints202206.0317.v1

Anti-proliferative and apoptotic properties are a large determinant to classify any compounds as anti-cancerous (Fantini et al., 2015). Results showed that CPPE and CPW extracts showed anti-proliferative effects against SW480 and HT-29 cells. While the CPW extracts exerted a cytotoxic effect on SW480 cells only at concentrations greater than 100µg/ml, the CPW extracts exerted a dose-dependent cytotoxic effects even at the lowest concentration (25ug/mL) used in this study. To confirm, if these cytotoxic effects observed on cancer cells are mediated by apoptosis, an APO Percentage assay was used. APO Percentage assay utilises the flip-flop translocation of phosphatidylserine demonstrated by showing by morphological appearance (Li, Fan, & Su, 2009; Pyrshev, Yesylevskyy, Mély, Demchenko, & Klymchenko, 2017).CPPE and CPW extracts induced apoptosis evidenced by the morphological changes in both the cell types. Quantitative analysis of this apoptotic activity further confirmed the apoptotic morphological observations. A dose-dependent increase in the percentage of cells undergoing apoptosis was observed post treatment with CPW and CPPE extracts. When treated with concentrations at and above 250 ug/ml of CPW and CPPE extracts, more than 50% HT-29 and SW480 cells underwent apoptosis when compared to the control. These results suggest that water in which the chickpeas are cooked contains bioactive compounds, leached out during the cooking process, can induce apoptosis in cancerous cells.

After the apoptosis is activated, cancer cells begin to acquire metastatic properties after tumour formation to maintain a suppressive environment. Metastasis determines the extent of cancer spread in tissue and organs (Fares, Fares, Khachfe, Salhab, & Fares, 2020). Metastasis by migration is a hallmark of cancer spread, orchestrated by specific cells surface proteins, the matrix metalloproteinase (MMPs). Amongst all the MMPS identified, MMP-2 and MMP-9 mediated signalling are the most crucial members for cancer migration and metastasis (Gu et al., 2021). HT-29 cells express MMPs 2, 7 and 9 (Lima, Mota, Monteiro, & Ferreira, 2016) and was therefore selected for cell migration assay in this study. Results from the scratch migration assay showed that both CPPE and CPW inhibited the cell migration when compared to the untreated cells. Studies investigating the anti-cancer properties of chickpeas are limited and are based primarily on the proteins and their hydrolysates (Faridy et al., 2020). Chickpea protein hydrolysates when fed to azoxymethane-induced carcinogenic mice, significantly reduced the neoplastic lesions in the colon (Sánchez-Chino et al., 2019). A study by (Lima et al., 2016) showed that albumin, but not the globulin chickpea fractions significantly slowed down the migration of HT-29 cells, an indication that chickpea extracts do have potential bioactivity to reduce cancer cell migration. However, there are no studies to date that have demonstrated the ability of CPW to reduce the migration of cancer cells.

Recent studies have investigated the composition, properties and use of CPW also known as aqua-faba. The CPW is gaining a lot of interest particularly in the vegan community to be used as an egg replacer due to its foaming and emulsifying properties (He, Meda, Reaney, & Mustafa, 2021; Mustafa, He, Shim, & Reaney, 2018). With the increasing consumer interests in CPW, several chickpea cultivars have been investigated to produce the aqua-faba with superior emulsifying properties (Echeverria-jaramillo et al., 2021). A study by (Meurer, de Souza, & Ferreira Marczak, 2020) used ultrasound technology to improve the foaming and emulsifying activity of CPW. All these aforementioned studies and findings from this study point to the increasing use of CPW as a value-added product for commercial use.

It is worth noting that there are few limitations to this study. The findings observed in this study are based on an *in vitro* model. Although the cell types used are very widely used and established colon cancer cell lines, not all hall marks of cancer pathways can be mimicked using a cell culture model. To account for any differences observed in cancer pathways, we have utilized two commercially available colon cancer cells in this study. A better profiling using mass spectrometry would have identified the bioactive compounds particularly in the CPW.

5. Conclusion

In summary, the results observed in this study demonstrate that both the chickpeas and the chickpea water contain bioactive compounds with anti-cancer activity. It is evident that bioactive compounds present in CPPE and CPW target pathways of cancer progression and development by inducing apoptosis and alleviating metastasis. Additionally, the water used for cooking chickpeas harbors beneficial bioactive compounds and should not be discarded after cooking. Future research using animal models is warranted to determine if anti-cancer properties observed in this study translates *in vivo*.

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Conflicts of Interest: The authors declare no conflict of interest.

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