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Commercial Lentils (*Lens culinaris*) Provide Antioxidative and Broad-spectrum Anti-cancerous Effects

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ABSTRACT

Background: Interest is growing surrounding functional foods, which provide health benefits in addition to nutritional value. In particular, there is a focus on pulse crops which contain high levels of polyphenolics, such as lentils.

Methods: In this study, polyphenols were extracted from five *L. culinaris* varieties and characterized by both their antioxidant profile and cytotoxic activity.

Result: Hulled varieties had a high content of secondary metabolites (>70 mg GAE/g), while all varieties demonstrated high antioxidant potential (1570-2020 Oxygen Radical Absorbance Capacity index). Furthermore, all extracts showed significant cytotoxicity against H9C2, HepG2, A549 and Calu-1 cancer cell lines. This recommends further investigation into the specific compounds present in *L. culinaris*, which could potentially be exploited for their anti-cancer activity.

Key words: Antioxidant capacity, ORAC, Polyphenols.

INTRODUCTION

Oxidative stress arises from malfunctioning internal antioxidant processes leading to range of pathophysiological conditions, primarily neurodegenerative disorders (Cassidy *et al.* 2020), biological ageing (Khansari *et al.* 2009) and cancer (Reuter *et al.* 2010). The economic and social burden of cancer continues to rise, with over 1.6 million new cases of cancer in 2016 in the United States alone (U.S. Cancer Statistics Working Group 2019). Invasive surgeries, radiotherapy and chemotherapy carry significant morbidity risks; hence prompting interest in alternative therapies (Keene *et al.* 2019). The rationale behind the phytotherapeutic approach against cancer is to modulate signal transduction pathways to substantiate anti-tumour immunity.

In addition to their high protein content (20-30%) (Khazaei *et al.* 2019), lentils contain a range of bioactive components, including lectins, hemagglutinins, phytates, oxalates, polyphenols and saponins displaying antioxidant, anti-inflammatory and anti-tumour properties (Margier *et al.* 2018). In the present study, *in vitro* cytotoxicity assays confirm apoptosis-mediated anti-cancerous properties of commercial lentil varieties at biologically relevant concentrations.

MATERIALS AND METHODS

Chemicals

Fetal bovine serum (FBS) and Dulbecco's minimum essential medium (DMEM) were purchased from Gibco, USA. All solvents were purchased from Sigma-Aldrich (St. Louis, USA) and were of analytical grade. Milli-Q® water was used where applicable.

Plant material

Lentil varieties were purchased from Ballarat (Victoria) retail supermarkets. Red and Yellow split lentils (McKenzie brand) were dehulled; Organic Green whole lentils (Life force Organic brand), Premium French and Whole Green lentils (McKenzie brand) were hulled. Experiments were conducted at the School of Health and Life Sciences, Federation University, Ballarat, Victoria, Australia in year 2017-18. Phosphatidylserine translocation assay was performed at Charles Sturt University, New South Wales, Australia.

L. culinaris crude extract (LcCE)

Samples were grounded and suspended in 5 time their volume of methanol:acetic acid (95:5 v/v). Polyphenols were extracted by shaking at 220 rpm, 25°C for 20 min, followed by centrifugation (10,000 rpm, 10 min) and collection of the supernatant. The pellet was further extracted twice, as above. Combined supernatants were rotary evaporated (Buchi R-200) to dryness at 27°C, reconstituted in distilled water (1:10 ratio w/v), filter-sterilized (0.22µm) and stored at -80°C. Before use, LcCE was freeze-dried (Christ Alpha 2-4LD Plus) and desiccated at -20°C.

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Antioxidant activity

Trolox equivalent antioxidant capacity (TEAC) was analysed using the CUPRAC method as described by Johnson *et al*. (2020a). Briefly, 1 ml of copper (II) chloride (10 mM), 1 ml of ammonium acetate buffer (1 M; pH 5.0), 1 ml of Milli-Q water and 1 ml of 5 mM neocuproine (prepared in absolute ethanol) were reacted with 100 µl of LcCEs (dark, 50°C, 30 min) prior to spectrophotometric quantification at 450 nm. Trolox standard curve (R^2=0.9991) was used to report results as Oxygen radical absorbance capacity (ORAC). TEAC in µmol Trolox/100g sample was expressed as ORAC in this study.

Total phenolic content

Total phenolic content (TPC) was measured as described by Özgen *et al*. (2010). Briefly, 400 µl of LcCE was reacted with 2 ml of 1:10 diluted Folin-Ciocalteu phenol reagent and incubated (dark, 10 min) prior to the addition of 2 ml of 7.5% (w/v) sodium carbonate (dark, 40°C, 30 min). TPC values were spectrophotometrically quantified at 760 nm as equivalents of gallic acid/ gram of sample (mg GAE/g) (R^2=0.9994).

Cell culture

Murine cell lines of C2C12 and H9C2 (myoblasts) and human lines of A549 and Calu-1 (lung), HepG2 (liver), SK-N-BE-2 (neuroblast) and Caco-2 (colon) were procured from the American Tissue Culture Collection (Manassas, USA). Cells were grown in complete DMEM (comprising DMEM with glucose, L-glutamine, sodium pyruvate; 10% FBS; and 100 U/ml penicillin-streptomycin) in T75 flasks at 37°C, 5% CO_2, until attainment of 80% confluency. After trypsinisation (5 min) and centrifugation (2500 rpm; 10 min), the cell pellet was washed thrice with warm DPBS (pH 7.2±0.2) and the cell count adjusted to 5×10^4 cells/ ml in complete DMEM. 100 µl of cell suspension was seeded into flat-bottom Corning® Costar® 96-well plates and incubated overnight.

Cytotoxicity assay

The cells (as grown above) were washed thrice with DPBS and treated with various LcCE concentration gradients (solubilised in complete DMEM containing 0.5% FBS). After incubating cells at 37°C for 72 h, 20µl of MTS tetrazolium compound was added to each well and incubated (37°C, 4 hrs, 5%CO_2). NADPH-dependent oxidoreductase activity was quantified spectrophotometrically at 490nm (*Multiskan*™ FC *Microplate* Photometer). The IC_{50} values were determined as the LcCE concentration causing 50% growth inhibition.

Phosphatidylserine translocation (PST) assay

Following LcCE treatment (0 mg/ml, 1 mg/ml and 2 mg/ml) for 72 h, the spent medium was replaced with 100µl DMEM (with no phenol red) containing 5% FBS and APOPercentage dye (Biocolor, UK). The cells were incubated for 30 min at 37°C, 5% CO_2. After washing twice with PBS and incubation (37°C, 5 min) the cells were suspended in 10 µl trypsin, followed by addition of 100 µl dye release reagent. Apoptosis was recorded spectrophotometrically at 550nm after shaking (10 min, 120 rpm) and microscopically (Nikon Eclipse Ti-U) to confirm morphological hallmarks of apoptosis.

RESULTS AND DISCUSSION

The global rise in cancer incidence has driven increased exploration of cancer preventive strategies, with phytotherapy emerging as one means of attenuating cancer risks. Hence methanolic LcCEs from the five commercial *L. culinaris* varieties were evaluated for ORAC, TPC and cell toxicity to elucidate efficacy of LcCEs against cancer cells.

Antioxidant capacity and total phenolic content

The ORAC of the LcCEs represented the sum of lipid-soluble hydroxycinnamic acid molecules and water-soluble phenolic antioxidant molecules. The median ORAC score was 1824, with virtually no difference between split (1835) and unsplit lentils (1839). Organic green lentil had the maximum ORAC score (2023), while Premium French had the lowest ORAC score (1573) (Fig 1). These results were comparable to literature mean values of 100-1970 ORAC for methanolic extracts of beans and lentils (Carlsen *et al.* 2010); 4310-4610 ORAC for whole green and red lentils and 2520 ORAC for dehulled red lentils (Agil *et al.* 2013). The range of ORAC values observed among hulled and dehulled varieties could be due to abiotic growth conditions of the lentil varieties (Pharmawati and Wijaya, 2019).

The TPC values varied from 62.8 mg GAE/g for yellow lentils to 102.0 mg GAE/g for whole green lentils (Fig 1). These results were in alignment with literature, where phenolic contents of 58.0 ± 1.4 and 67.6±1.7 mg catechin equivalents/g have been reported for red and green lentils, respectively (Moïse *et al.* 2005). In general, unsplit hulled lentils showed higher TPC values (median value of 89.8 mg GAE/g; n=3 varieties) as compared to the lower values for

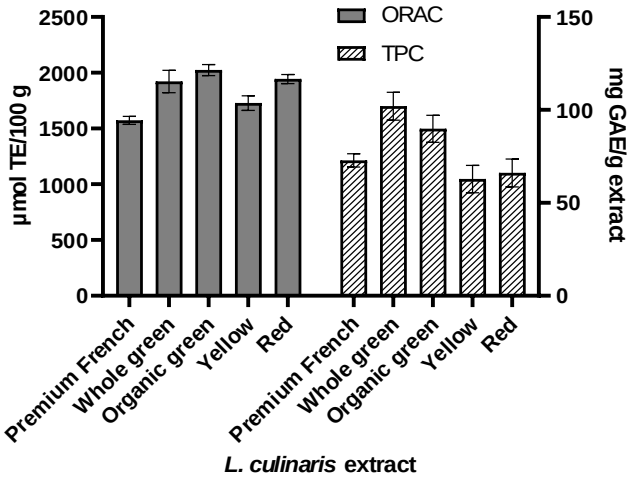

Fig 1: Total antioxidant capacity as ORAC (µmol TE/100g) and total phenolic content (mg GAE/g) in different LcCEs (n = 4 replicates for each).

Table 1 : Evaluation of the cytotoxic potential of LcCEs against A549, Calu-1, HepG2 and H9C2 cell lines at given concentrations.

Cell lines	Conc (mg/ml)	Percent growth Inhibition (%) of cancer cells by LcCEs				
		Premium French	**Whole Green**	**Organic Green**	**Yellow**	**Red**
A549	**0.1**	10.57	2.78	-6.04	10.74	5.263
Calu-1		-63.01	0.09	-29.4	-37	-62.58
HepG2		-27.99	-	-20.21	-15.4	1.23
H9C2		-1.6	-0.1	-1.2	8.48	-3.39
A549	**0.2**	12.8	2.84	12.88	14.17	14.59
Calu-1		0	0.12	2.5	-18.15	-23.9
HepG2		-26.54	-	-2.5	-10.4	5.3
H9C2		10.71	-0.07	5.2	12.43	1.223
A549	**0.5**	31.9	3.89	13.44	25.67	21.38
Calu-1		39.69	0.09	47.29	-5.39	38.6
HepG2		-15.64	-	-1.5	-5.6	17.9
H9C2		16.58	-0.02	14.08	16.84	4.27
A549	**1**	60.82	4.33	70.66	50.34	70.1
Calu-1		57.92	0.9	58.62	38.79	52.43
HepG2		21.59	-	53.6	59.3	60.96
H9C2		20.87	-0.1	14.19	23.75	10.52
A549	**2**	88.04	6.88	83.4	75.29	84.15
Calu-1		68.5	1.2	62.68	55.09	64.49
HepG2		40.33	-	70.12	80.12	83.46
H9C2		24.35	0.009	14.25	34.47	10.61

The bars indicate the extent of cell metabolic inhibition as a function of NADPH activity reduction and hence inhibition of cell growth.

the split (dehulled) varieties of yellow and red lentils. The median value for all LcCEs was 64.4 mg GAE/g.

Although ORAC and TPC are generally correlated in most matrices (Djordjevic *et al.* 2011; Guleria *et al.* 2013; Johnson *et al.* 2020b), no significant correlation between the TEAC and TPC of the LcCEs was observed here (Pearson r = 0.490; p > 0.05). This is likely due to the small sample size (n=5), given that our primary focus was on anti-cancer activity. However, further investigation into the separation and identification of phenolics from lentils using chromatographic techniques such as HPLC is warranted, as the Folin-Ciocalteu method may not always be representative of the quality or quantity of phenolic constituents present (Guleria *et al.* 2013). For example, reducing agents such as L-ascorbic acid and sulphur dioxide may react with the reagents and result in an overestimation of the TPC (Yu *et al.* 2002). The TEAC could also be measured with other less selective reduction methods, such as the ferric reducing antioxidant potential (FRAP) (Djordjevic *et al.* 2011).

Cytotoxicity assay

Cell cytotoxicity was estimated as NAPDH-dependent oxidoreductase activity in H9C2 (rat cardiomyocytes), HepG2 (human hepatocarcinoma cells), A549 and Calu1 (human lung cancer cells) cell-lines. To observe cytotoxicity, the IC_{50} of different LcCEs was determined by subjecting different cell lines at 80% confluency to a LcCE concentration gradient of 0-2mg/ml. Cell cytotoxicity was observed to increase along the increasing concentration gradients of all five LcCEs (Table 1). A549 cells were observed to be the most susceptible whereas H9C2 cells were the least susceptible cell line as the IC_{50} was not achieved at the highest treatment concentration of 2mg/ml.

Red and Premium French LcCEs demonstrated IC_{50} value of 1mg/ml concentration on A549 cells as compared to whole green LcCE ($p < 0.0001$ for both). Another study also showed comparable IC_{50} values between *Glycine soja* extracts (from the same family as lentils) and cisplatin (broad-spectrum chemotherapy medicine) on A549 cells (Amaani and Dwira, 2018).

Table 2: Percent apoptosis in different cell lines induced by the lentil extracts.

cell lines	Conc.	Percent apoptosis (%) in cancer cells				
		Premium French	Whole Green	Organic Green	Yellow	Red
A549	1mg/ml	60.84	61.92	62.03	58.37	69.9
Calu-1		50.33	31.49	39.1	57	63.19
H9C2		38.39	18.13	32.49	38.32	51.14
C2C12		70.83	69.46	58.79	77.06	71.64
Caco2		40.9	1.6	42.86	54.39	76.36
SK-N-BE-2		50.4	35.9	54.13	76.5	69.9
A549	2mg/ml	71.39	64.34	63.93	66.44	84.32
Calu-1		55.17	42.67	67.2	68.52	80.25
H9C2		34.76	59.59	33.9	74.9	78.57
C2C12		55.78	47	55.78	61	92.36
Caco2		63.64	14.09	76.33	73.16	85.13
SK-N-BE-2		79.2	65.5	93.96	98.59	96.29

The number of apoptotic cells was expressed as the percentage of absorbance of LcCE treated cells with respect to non-apoptotic control cells (data not shown).

Whole green LcCE demonstrated insignificant cytotoxicity against A549 cells with only 6.88% inhibition even at 2mg/ml concentration. However, all other LcCEs demonstrated steady increase in the percentage growth inhibition of A549, Calu-1, HepG2 and H9C2 cells along concentration gradient (Table 1), with IC_{50} values achieved at 1mg/ml concentration. A549 cells were most susceptible to all LcCEs, followed by Calu-1 and then H9C2 cells (Table 1). Xu and Chang (2012) and Johnson *et al*. (2020a,b) reported that beans (e.g. black, pinto and red kidney beans), black soybean, lentil, adzuki bean, *Vicia faba* and mungbean demonstrate stronger antioxidant capacities and cancer cell proliferation inhibition, as compared to other common pulses. Although phenolic profiling of the LcCE was not conducted, the anti-cancer activity observed in this study could be due to compounds such as procyanidin and prodelphinidin dimers and trimers, gallate procyanidins, kaempferol derivatives, quercetin glucoside, luteolin derivatives and p-coumaric acid. These compounds have been reported in lentils and also inhibit proliferation of cancer cells (Ganesan and Xu, 2017).

Furthermore, microscopic observations confirmed significant morphological changes upon exposure to LcCEs. Morphological changes progressively involved cell counts showing cell shrinkage, rounding and membrane blebbing which indicated late or early necrosis. To confirm the underlying apoptotic events in cells, cell membrane phosphatidylserine translocation to the cell surface was used as a hallmark of cell necrosis.

Phosphatidylserine translocation (PST)

Phosphatidylserine translocation is sequential to DNA fragmentation during apoptosis. DNA fragmentation triggers the inactivation of flippase and activation of scramblase which causes PST (Segawa and Nagata, 2015). PST leads to dye uptake by cells undergoing apoptosis until cells reach the blebbing stage. In this study, the apoptotic potential of LcCEs was reported w.r.t. H_2O_2 as positive control. H_2O_2 has a known necroptosis effect on cells *via* caspase-3 and caspase-9 mediated apoptosis (Gutiérrez-Venegas *et al.* 2015).

The spectrophotometric analysis of A549, Calu-1, H9C2, Caco2, C2C12 and SK-N-BE-2 cells demonstrated that all the cells underwent apoptosis. Broadly, unsplit lentil varieties demonstrated higher apoptotic potential as compared to split varieties. Extracts from red lentils showed the highest apoptotic potential at concentrations of 1 mg/ml (51.14% to 76.4%) and 2 mg/ml (78.6% to 96.3%) against all cell types (Table 2). Extracts from unsplit lentils, primarily whole green lentils, showed significantly lower apoptosis potential ($p<0.01$) against all cell lines, except for C2C12 cells, which were equally susceptible to all LcCEs at 1 mg/ml concentration. Subsequently, the lower apoptosis potential of whole green lentil extracts corresponded to the low cytotoxic effects as previously noted (Table 1). It was also noted that SK-N-BE-2 cells demonstrated a high apoptotic response to all LcCE treatments (Table 2). The cytotoxic potential of the LcCEs could be due to the activation of caspase-3 mediated apoptosis (Busambwa *et al.* 2016).

With such reliance of cancer cells on reactive oxygen species (ROS) such as peroxides, it follows that if antioxidant phytochemicals can scavenge ROS molecules, the oxidative stress-responsive genes can be suppressed and consequently cancer cell proliferation inhibited.

CONCLUSION AND FUTURE PERSPECTIVES

The phytochemical characterization and cell cytotoxicity assay substantiate the candidature of *L. culinaris* as a

potential anti-cancerous food. Analysis of TPC, TEAC and cytotoxicity assays showed that LcCEs had anti-proliferative effect on all the tested cells wherein A549 cells were the most susceptible and H9C2 were the least susceptible cells. Despite having high TPC values, whole green LcCE had no significant cytotoxic effects. Underlying apoptotic events in cells were confirmed by the cell membrane PST assay. In general, the higher apoptotic potential was evident in unsplit, hulled lentil varieties as compared to split varieties.

Further studies are warranted using LcCEs in animal models of Ehrlich ascites carcinoma (EAC) cells. The latter will confirm restoration of CD4+/CD8+ T-cell proliferation, expansion of memory T-cells and reduced expression of tumour-derived Tregs as hallmarks of anti-tumour immunity. Subsequently, further screening of the functional polyphenols causing cytotoxicity and apoptosis can be performed before future animal and clinical trials.

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