Pharmacogn. Commn. 2021;11(4):210-213.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Short Communication

Australian Ethnomedicinal Plant Extracts Promote Apoptosis-Mediated Cell Death in Human Hepatocellular Carcinoma *in vitro*

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ABSTRACT

Introduction: Hepatocellular carcinoma (HCC) is the leading cause of primary liver cancer with its prevalence continuing to rise. Although the number of cases continues to rise in both developing and developed countries, prognosis remains poor due to a lack of successful treatments. Inspired by the prospect of developing complementary medicines for this condition, we explore several native Australian plants for anti-carcinogenic activity, especially against HCC. **Methods:** Cytotoxicity assays against HCC cell lines were conducted using various plant extracts. Biochemical profiling of the plant species was conducted for total phenolics and antioxidant capacity, while reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the active apoptotic pathways. **Results:** *Westringia fruticosa* and *Prostanthera ovalifolia* (small-leaved variety) had high antioxidant (410 and 227 mg/g, respectively) and phenolic contents (72.7 and 42.7 mg/g, respectively). *P. ovalifolia* (small-leaved variety) demonstrated the greatest cytotoxic activity against HepG2 cells (IC_{50} 4.61 ± 0.98 µg/mL) followed by *Solanum laciniatum* leaves (11.79 ± 0.43 µg/mL) and fruit extracts (ripe, unripe) (14.85 ± 1.80 and 19 ± 1.32 µg/mL, respectively). RT-PCR results confirmed apoptotic events in HepG2 cells, exposed to ripe and unripe *S. laciniatum* fruit extracts, via caspase-3 pathway. The highest apoptotic induction occurred after 8 hr. Compared to unripe fruits, ripe fruits induced a greater level of apoptosis, as evidenced by a 73 % higher level of caspase-3 mRNA expression and 22 % lower IC_{50} value. **Conclusion:** With further investigation, these fruits may provide a valuable source of anticarcinogenic compounds for use as chemotherapeutic or complementary therapies.

Key words: Bioactive Compounds, Antioxidant Activity, Phenolics, Chemotherapeutic, Anti-tumour activity.

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DOI: 10.5530/pc.2021.4.41

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide,[1] with therapies limited to liver transplantation or surgical resection.[2] Consequently, complementary and alternative medicine provides new avenues for therapeutic management by reducing the risk and progression of cancer.

Oxidative stress and lipid peroxidation, resulting from viral hepatitis, metabolic disorders, or lifestyle, triggers somatic mutations in HCC.[3] However, treatment with polyphenols such as Juglanthraquinone C can restore peroxiredoxin-4 expression and antioxidant response element (ARE) in HCCs.[4,5] Other polyphenols are well known for the prevention of cancer, cardiovascular, and neurodegenerative diseases.[6,7] Following from previous work highlighting the high polyphenolic content of several native Australian plants, namely *Solanum laciniatum, Prostanthera ovalifolia* and *Westringia fruticosa*,[8] this study evaluates their therapeutic potential against HCC using a human hepatoma-derived cell line (HepG2). *Hibiscus sabdariffa* leaves, previously reported to have anti-proliferative potential,[9] were included for comparison.

MATERIALS AND METHODS

Plant samples

Solanum laciniatum leaves (L), unripe (U) and ripe fruit (R); *Prostanthera ovalifolia* small (S) and large (P) leaved variety; and *Westringia fruticosa* leaves (W) were collected from Ballarat, Victoria. *Hibiscus sabdariffa* leaves (H) were obtained from a commercial market in Brisbane, Queensland. Except where specified, all analyses were conducted in quadruplicate.

Preparation of plant extracts

Dried, ground plant material was extracted in methanol (1:20 w/v) at 220 rpm and 4°C for 15 hr.[10] After filtering (Whatman No. 4) and concentration in a rotary evaporator (27°C), extracts were reconstituted in 20mL water, syringe filtered (0.22 μm) and freeze-dried (Christ Alpha 2-4 LD Plus operating at -80°C).

Total phenolics and antioxidant capacity

The total antioxidant capacity (TAC) of the sample extracts was determined using the cupric reducing antioxidant capacity (CUPRAC) method as described by Johnson *et al.*[11] with the TAC derived as equivalent absorbance of Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid) in ethanol solution at 450 nm (R^2 = .99). The total phenolic content (TPC) was determined using the Folin-Ciocalteu method,[11,12] quantified as a function of the equivalent absorbance of gallic acid at 760 nm (R^2 = .99).

Cytotoxicity assay

Human hepatocarcinoma cells (HepG2 HB-8065™) cells were procured from American Type Cell Culture (ATCC; Manassas, VA) and cultured in complete Dulbecco's Modified Eagle Medium (DMEM) constituting DMEM-GlutaMAX, 10% v/v FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. After harvesting cells (using 0.05% (w/v) trypsin–EDTA) and suspending them in complete DMEM at a concentration of 5×10^5/ml, 100µL was seeded into flat-bottom Corning® Costar® 96-well plates after dye exclusion testing, and incubated (24 hr, 37°C, 5% CO_2). After replacing the culture medium with complete medium containing 0.5% v/v FBS and incubating (4h, 37°C, 5% CO_2), selected wells were subjected to

concentrations gradient of plant extracts (0-500 µg/mL) and incubated for 24hr, 48hr, and 72hr to evaluate time-dependent cell cytotoxic potential.

To determine the effective inhibitory concentrations (IC_{50} values) of the plant extracts, 20 µl of MTT reagent was added to the above-treated cells and they were incubated (4h, 37°C, 5% CO_2) according to the manufacturer's protocol. Cytotoxicity was quantified spectrophotometrically at 570 nm (Multiskan™ FC Microplate Photometer; ThermoFisher Scientific, Australia).

Determination of Apoptotic-Related Gene Expression

To investigate the induction of apoptosis in HepG2 cells, the mRNA expression level of *caspase*-3 was analysed by semi-quantitative RT-PCR. HepG2 cells (3×10^6/ml) were cultured in 96-well plates and incubated (24hr, 37°C, 5% CO_2) in complete DMEM. The cells were washed twice with DPBS (37°C, pH 7.2), mixed with complete DMEM (2 mL) containing 0.5% v/v FBS and incubated (4hr, 37°C, 5% CO_2). Hep2 cells were then treated at different incubation periods (0-24 hr) with the previously determined IC_{50} concentrations of R and U extracts. Subsequently, RNA was isolated using the TRIzol Reagent according to the manufacturer's instructions and stored in RNase-free water at −80°C.

After DNase I treatment of RNA, cDNA was synthesised using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. The cDNA synthesis program was set according to the manufacturer's instructions. Initially, 20 ng of cDNA template was subjected to PCR amplification using human *ACTB* (house-keeping gene) and casp-3. A Power SYBR Green PCR Master Mix (Applied Biosystems) was used as the fluorescence source. Primers were designed with the Primer 3 software using the sequence information listed at the National Center for Biotechnology Information. The primer sets used were: human *Casp*-3 reverse primer 5' GGC AGG CCT GAA TAA TGA AA 3' with Tm 54; human *Casp*-3 forward primer 5' TCA CAG CAA AAG GAG CAG TT 3' with Tm 53.9; *ACTB* F 5' CGC GAG AAG ATG ACG CAG AT 3' with Tm 56.3; and *ACTB* R 5' GAG TCC ATC ACG GAT GCC AGT 3' with Tm 54.4. The optimized PCR amplification program comprised an initial denaturation step at 94°C for 2 min, followed by denaturation at 94°C for 45 sec, annealing at 56°C for 1 min, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min, with 40 amplification cycles.

Statistical analysis

Excel (Microsoft Office Suite) was used for standard calculations of gene expression analysis along with the calculation of IC_{50} values, *p*-values and standard deviations. Graphs were drawn in GraphPad Prism. Where applicable, plots show the mean ± one standard deviation.

RESULTS AND DISCUSSION

The TAC and TPC of all plant extracts is given in Figure 1. For *S. laciniatum*, the leaf extracts showed significantly higher TAC and TPC values compared to ripe fruits, which in turn were lower than ripe fruit. TAC and TPC values were strongly correlated (R^2 = .983).

The *S. laciniatum* leaf and *P. ovalifolia* (small-leaf) extracts demonstrated significant growth inhibition of HepG2 cells along the concentration gradient at 48-72hr incubation (Table 1). Consequently, the IC_{50} values of all plant extracts against HepG2 cells were determined after 72hr incubation (Table 1). The P, H and W extracts demonstrated no cytotoxicity at the highest tested concentration (500 µg/ml). The S extract demonstrated the highest IC_{50} value (4.61±0.98 µg/ml), suggesting significantly different phytochemical composition to the large-leaved variety (which possessed an IC_{50} >500µg/ml). The anti-proliferative IC_{50} levels of extracts L, U and R ranged from 11.8-19 µg/ml.

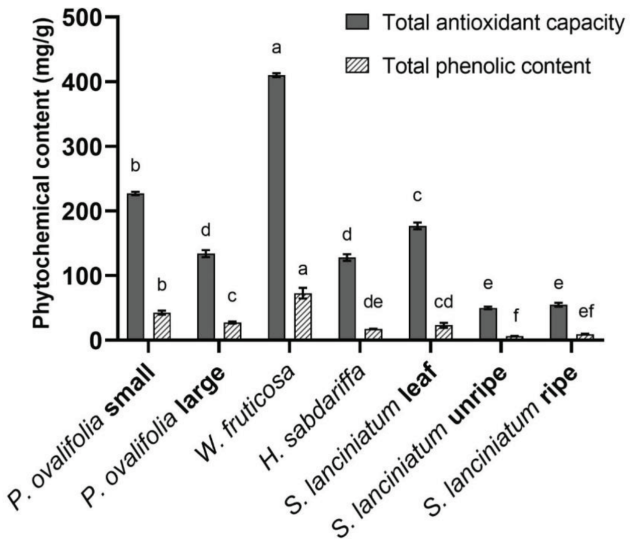

Figure 1: Total antioxidant capacity and total phenolic content of the plant extracts, given as Trolox equivalents and gallic acid equivalents, respectively (mg/g for both; *n*=4). Error bars show mean ± 1 standard deviation. For each parameter, samples containing different letters were significantly different from one another according to a one-way ANOVA followed by post hoc Tukey testing at α = 0.05.

Caspase-3 mediated apoptosis

The time-dependent molecular expression of *casp-3* (caspase 3 gene) in HepG2 cells treated with U and R extracts was quantified by reverse transcription polymerase chain reaction (RT-PCR) (after 0hr, 4hr, 8hr, 12hr, and 24hr). Microscopical examination revealed apoptosis morphotypes corresponding to cellular swelling and chromatin condensation. *Casp*-3 expression gradually increased (after 4h incubation), peaked markedly at 8h at levels 9.17-fold (unripe fruit) and 15.9-fold (ripe fruit) higher than initial expression levels, then decreased again (Figure 2). This confirmed that treatment of HepG2 cells with *S. laciniatum* fruit extracts induced *casp*-3-mediated early phase apoptotic events, resulting in cell death.

Exogenous antioxidants from ethnomedicinal sources are well-known for anti-lipid-peroxidation activity. In this study, S, L, R, and U plant extracts from *P. ovalifolia* and *S. laciniatum* demonstrated strong potential for the prevention of HCC, as evidenced by their IC_{50} values for cytotoxicity and *casp-3* expression for apoptosis. The IC_{50} values of S and L extracts were significantly reduced 4.8-fold ($p<0.01$) by reducing the incubation time from 72hr to 48hr. After 72hr incubation, the IC_{50} values of these four extracts (S, L, R, and U) were below 20 µg/mL, evincing strong cytotoxic potential, as defined by the American National Cancer Institute.[13]

S. laciniatum has previously shown anti-proliferative activity against lung, liver, brain, lymphoblastic leukemia and breast carcinoma cell lines,[14] attributed to the general cytotoxic potential of glycoalkaloids.[15] The bioactive components responsible for cytotoxic potential of *P. ovalifolia* (S) and *S. laciniatum* (L, R, U) extracts are not yet reported, underscoring the novelty of this study. Generally, the antioxidant potential of phytochemicals scavenges reactive oxygen species *in vivo*, hence preventing the genotoxic effect associated with oxidative stress.[6,8,16] However, TAC and TPC did not correlate with cytotoxic activity in this study. Extracts W, P and H (high TAC/TPC) showed no cytotoxicity, while extracts R and U (low TAC/TPC) showed strong cytotoxicity and apoptotic activity. The reason for this discrepancy lies in their polyphenolic spectrum of phenols, flavonoids, flavanols, anthocyanidins and tannins,

Table 1: IC_{50} values of the plant extracts against HepG2 Cells at 24, 48, and 72 hr of incubation. Values given as mean ± standard deviation ($n = 4$).

Plant Extract	Incubation Times (h)	IC_{50} Values (µg/ml)
S. laciniatum leaf	24	>500
S. laciniatum leaf	48	55.1 ± 2.20*
S. laciniatum leaf	72	11.79 ± 0.43**
P. ovalifolia small-leaved variety	24	>500
P. ovalifolia small-leaved variety	48	187.25 ± 3.04*
P. ovalifolia small-leaved variety	72	4.61 ± 0.98***
P. ovalifolia large-leaved variety	72	>500
W. fruticosa leaf	72	>500
H. sabdariffa leaf	72	>500
S. laciniatum unripe fruit	72	19 ± 1.320
S. laciniatum ripe fruit	72	14.85 ± 1.80

*$p < 0.01$; **$p < 0.001$; ***$p<0.0001$ (72 hr incubation compared with 48 hr incubation)

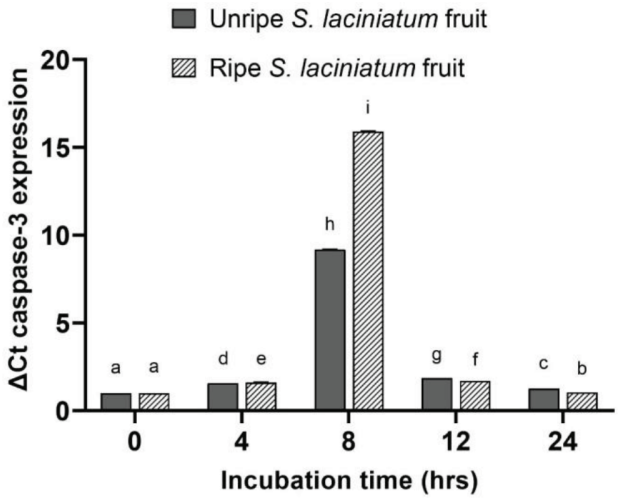

Figure 2: Temporal changes in caspase-3 mRNA expression of HepG2 cells treated with *S. laciniatum* extracts from ripe and unripe fruits. Error bars show mean ± 1 standard deviation. Bars annotated with different letters were significantly different from one another according to a one-way ANOVA followed by *post hoc* Tukey testing at α = 0.05.

that provide higher TAC scores[17] but may be ineffective against HepG2 cells. For example, high-antioxidant *Opuntia stricta* extracts are cytoprotective against HepG2 cells.[18,19]

CONCLUSION

Molecular data demonstrating the activation of the *casp*-3 apoptosis cascade by R and U extracts of *S. laciniatum* underscores the therapeutic potential of these extracts against HCC. These plant extracts are attractive candidates in designing and developing alternative therapies for cancers. Further quantitative studies are required to identify the specific antitumor or immunity-boosting phytochemicals present in this species.

ACKNOWLEDGEMENT

The assistance of the Biomedical Sciences team, Federation University, is gratefully acknowledged.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ARE: Antioxidant response element; **ATCC:** American Type Cell Culture; **cDNA:** complementary DNA; **CUPRAC:** cupric reducing antioxidant capacity; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **H:** Hibiscus sabdariffa leaves; **HCC:** Hepatocellular carcinoma; **IC_{50}:** half-maximal inhibitory concentration; **L:** Solanum laciniatum leaves; **mRNA:** messenger RNA; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **P:** Prostanthera ovalifolia large-leaved variety; **PCR:** polymerase chain reaction; **R:** Solanum laciniatum ripe fruit; **RT-PCR:** reverse transcription-polymerase chain reaction; **S:** Prostanthera ovalifolia small-leaved variety; **TAC:** Total antioxidant capacity; **TPC:** Total phenolic content; **U:** Solanum laciniatum unripe fruit; **W:** Westringia fruticosa leaves.

SUMMARY

- Compounds derived from native Australian plants show great potential as anti-cancer agents
- 4 of the 7 plant samples showed some activity against hepatocellular carcinoma
- The greatest activity was found in the small-leaved variety of Prostanthera ovalifolia
- Ripe Solanum laciniatum fruit showed much higher apoptotic activity compared to unripe fruit
- The anti-cancer activity was confirmed to occur through activation of the casp-3 apoptosis cascade pathway

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Cite this article: Kiran S, Chew GS, Johnson JB, Mani JS, Wakeling L, Portman A, *et al*. Australian Ethnomedicinal Plant Extracts Promote Apoptosis-Mediated Cell Death in Human Hepatocellular Carcinoma *in vitro*. Pharmacognosy Communications. 2021;11(4):210-3.