

Can *Rhus Coriaria* be a Potential, Natural, Treatment for Prostate Cancer?

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Abstract

The defining hallmarks of cancer render tumours as an active and evolving tissue, capable of evading anti-cancer therapies. Highly metastatic cancers such as triple negative breast cancer, colorectal cancer and prostate cancer have some of the highest mortality rates owing to rapid growth, resistance to cell death and acquisition of invasive and angiogenic traits. Currently, there are no defined treatments for such cancers in averting metastasis and re-occurrence. Plant-derived phytochemicals are potentially invaluable sources of anti-cancer agents in combinational therapies. *Rhus coriaria*, also known as Sumac, is a common spice, which has extensive medicinal properties frequently referred to in traditional herbal remedies. Treating triple negative breast cancer and colon cancer cell lines with extracts of *Rhus coriaria* has shown profound and diverse anti-cancer effects previously. Here we used a variety of 'normal' and prostate cancer (PrCa) cell lines as our *in vitro* model in order to dissect the benefits of Sumac using a cell-based assay and informatic analysis. Our data clearly depicted increased killing effects of Sumac in comparison to Docetaxel and Vinblastine. However, normal cells were affected to a greater extent, than the cancer cells. Hence, our study demonstrates that the potential killing effect of the *Rhus coriaria*, on the growth of normal cell is equally adverse as on cancer cells. This may be due to exposure of cells to a heterogeneous mixture of hundreds of compounds at a time that could kill normal cells more adversely due to stress.

Keywords: Prostate cancer, *Rhus coriaria*, Treatment, Diagnosis

Introduction

Early diagnosis and intervention have successfully led to a steady decline in overall cancer deaths by about 1.5% per year since the early 1990s [1]. However, current strategies for targeting re-occurrence have transient durability. Complementary and alternative approaches such as herbal remedies and nutrition are of growing interest, particularly to breast cancer patients [2]. This has accompanied advances in the use of plant-derived phytochemicals as a source of clinically active anti-cancer agents with highly specific molecular targets. Such examples include Camptothecin, a potent DNA topoisomerase I inhibitor from *Camptotheca acuminata* and Combretastatins from *Combretum caffrum*, with anti-angiogenic properties effective against leukaemia and colon cancer [3].

Rhus coriaria (Sumac) is one of more than 250 species within the *Anacardiaceae* family [4]. Traditionally, the dark red fruits are dried and ground to produce a crimson sour spice commonly used in Middle Eastern cuisine [5]. In addition, extracts of Sumac have been used in the treatment of diabetes, anorexia, haemorrhage [5] and for wound healing [6]. Rapid profiling and isolation of phytochemical compounds within Sumac, by high resolution mass spectrometry, reveal its many pharmacological properties. Such active compounds include flavonoids, tannins and xanthons [7] all with known anti-microbial [8]; antifungal [9]; hypo-glycaemic [10]; and antioxidant and radical scavenging activities [11]. Iranian literature, dating back to 865-925 AD, addresses the anti-cancer properties of Sumac, prompting its use in the treatment of conjunctiva fibrovascular proliferation in ocular diseases [12] and recommending its consumption for cancer patients [13]. Recent research on triple negative breast

cancer (TNBC) cell lines is beginning to elucidate the anti-cancer impact of Sumac on cell cycle regulation, senescence, autophagy, anti-angiogenic and anti-metastatic effects.

Cell cycle

Novel studies using highly metastatic TNBC MDA-MB-231 cells have demonstrated the cytotoxic effects of Sumac ethanol extract (RCE) with compelling inhibition of several of the hallmarks of cancers, interfering with functional redundancy often adopted in aggressive and metastatic cancers [14]. MDA-MB-231 cells treated with RCE showed significant cell cycle inhibition and arrest in G₁ phase in a time and concentration-dependent manner [14]. In 48 hours, the G₁ population increased from 57% ± 2 in control cells to 67% ± 4 and 71% ± 0.1 in cells treated with 200 and 400 µg/mL RCE, respectively. Considerable growth inhibition following RCE treatment is likely due to effects on cell cycle activators and inhibitors, disrupting finely orchestrated transitions through the cell cycle. Cyclin D1, intrinsically induced by early mitogenic stimulation, was decreased to an almost undetectable level when cells were exposed to high concentrations of RCE, blocking G₁ progression in TNBC cells [14]. This was concomitant with the downregulation of proliferating cell nuclear antigen, PCNA, an auxiliary protein in DNA polymerase-δ [14], with additional temporal function at the G₁/S phase transition [15]. The fall in free Cyclin D1 and PCNA

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levels may be owing to the formation of an inhibitory complex between the two. Cyclin D1-PCNA complexes are markedly increased in senescent cells as DNA replication and proliferation are suppressed [16].

Senescence

The growth inhibitory effects of Sumac concurred with changes in MDA-MB-231 cells indicative of senescence. Despite antagonistic pleiotropic consequences of senescence, its induction could pose a significant barrier for tumour progression by blocking incipient cancer cell proliferation. Notably, after only 24-hour treatment with RCE, cells displayed typical morphological changes - flattening and almost doubling in size [14,17]. Subsequent staining for senescence associated- β -Galactosidase (SA- β -G) biomarker and expression assays were diagnostic of senescence [14]. The mechanisms by which Sumac induces senescence in TNBC and colorectal cancer cells remains unclear. However, it may be postulated that Sumac drives DNA damage response, to negotiate critical tumour suppressor mechanisms (Figure 1), leading to p53-dependent growth arrest [18]. In fact, RCE treated cells show elevated levels of phosphorylated γ H2AX, indicative of double-stranded breaks in the DNA, preceding any later signs of senescence [14].

RCE treated MDA-MB-231 cells had considerably reduced levels of phosphorylated pRb and c-myc expression, depictive of senescence. Concurrently, when exposed to 100 and 200 μ g/mL RCE, cells displayed increased levels of p21, suggesting

Sumac may, in part, function by promoting senescence [14]. p21 also functions to inhibit PCNA, comparable to Cyclin D1; prevent nuclear export of cyclin D1 [19]; and regulate proto-oncogene, c-myc, an exceedingly amplified oncogene [20]. However, subsequent time point assays showed there was in fact, a transient increase in p21 levels, which fell after 48 hours post-RCE-treatment [14]. Moreover, findings showed there was downregulation of p16 and p27 CDK inhibitors, discordant with the observed increase in cell cycle arrest [14]. Hence, the results show some level of inconsistency with little mechanistic explanation as of yet.

Autophagy and cell death

Principally, autophagy is a pro-survival response to maintain functional proteins and organelles and for the mobilisation of energy from macromolecules [21]. Paradoxically, defective autophagy is often concomitant with increased tumorigenesis. Hence, autophagy offers a way to modulate programmed cell death (PCD) in cancer cells [22].

RCE treated TNBC cells exhibited typical biomarkers of canonical autophagy with visible cytoplasmic vacuolation, formation of autophagosomes, autolysosomes, alongside swollen rough endoplasmic reticulum. Further assays showed increased Beclin-1, significant LC3II accumulation, and decreased p62 levels [14] associated with greater autophagy flux. The mechanism of Sumac induced autophagy remains speculative. However, the actions of other anti-breast cancer agents, such as capsaicin, exploit the activation of mitogen-activated kinases ERK1/2 and p38 in autophagy. Phosphorylated ERK1/2 and p38 are associated with autophagosome maturation and fusion with lysosomes, respectively. Indeed, Sumac led to sustained activation of both kinases, coinciding with LC3II accumulation [14].

Absence of proliferative recovery and decreased viable cell count suggest RCE treated cells had undergone cell death, yet there was minimal induction of apoptosis [14]. It is posited that Sumac inflicts anti-breast cancer effects through Type-II programmed cell death from excessive autophagy that renders irreversible cell atrophy and can ensue independently of apoptosis. Autophagy inducers have been identified as potent anti-cancer therapies and include many chemotherapies and radiation [23]. Similarly, Sumac exerts anti-colon cancer effects through inducing autophagic response [24]. However, characteristic signs of autophagic vacuolation and downregulation of p62 coincided with an unexpected loss of Beclin-1. Thus, contrary to TNBC cells, there was induction of non-canonical Beclin-1-independent autophagy in colon cancer cells, suggesting cell type specific autophagy responses to RCE. Further assays revealed Beclin-1 is targeted for proteasomal degradation. Strikingly, RCE stimulated an overall increase in intracellular ubiquitination- including that of mTOR, Akt, mutant p53 and caspase 3, preconditioning for targeted proteasomal degradation, in a time sensitive manner [24]. Notably, mTOR degradation occurred just 3 hours following treatment, accompanied by an increase in global ubiquitination. This may illustrate decreased mTOR activity as a means of activating ubiquitin-proteasome system (UPS). Furthermore, observed loss of mutant p53 in RCE treated TNBC cells may have occurred through the same mechanism [14].

RCE may exert anti-colon cancer effects by virtue of the functional interplay between the UPS and autophagy,

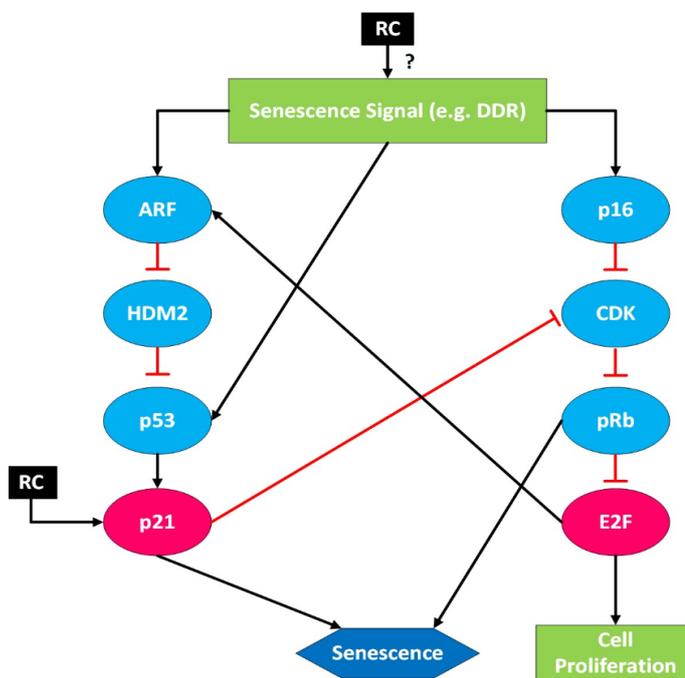


Figure 1. The role of p53 and p16-pRb pathways in promoting cellular senescence following senescent signals, showing their distinct effects and also connectivity. RC indicates where Sumac may impact to induce greater senescence in treated cancer cells. RC seemingly induces DNA damage response (DDR) which primarily engages the p53 pathway via alternative-reading frame protein (ARF), which binds the E3-ubiquitin-protein ligase, HDM2, blocking poly-ubiquitination and subsequent proteasomal degradation of p53. p53, and RC, induces expression of p21, a CDK inhibitor, preventing phosphorylation of pRb. Hypophosphorylated pRb prevents E2F dependent transcription, blocking G1 progression and inducing growth arrest in senescence [34].

coordinated by p62 and histone deacetylase 6 (HDAC6). This serves as a compensatory mechanism to cope with protein degradation exceeding the degradative capacity of the proteasome to facilitate in re-directing ubiquitinated proteins to autophagosomes [25]. Widespread ubiquitination and subsequent proteasomal degradation precipitates Beclin-1-independent autophagy, ultimately leading to a pro-death response, principally through Type-II programmed cell death. Apoptosis was detected secondary to Type-II PCD and to a lesser extent in RCE treated colon cancer cells [24].

Migration, metastasis and angiogenesis

Treatment with Sumac has shown to induce anti-migratory effects, reduce adhesion and invasive capabilities in TNBC cells [26]. RCE treated TNBC cells showed significantly reduced levels of MMP-9 and prostaglandin E2 (PGE2) while diminishing integrin-fibronectin interactions, resulting in attenuated migration of MDA-MB-231 cells [26]. Sumac may perhaps downregulate Akt in breast cancer through proteasomal degradation as in RCE-treated colon cancer cells, reducing epithelial-mesenchymal transition (EMT) and repressing metastasis [24]. Importantly, there was reduced adhesion to human umbilical vein endothelial cells (HUVECs) and to lung microvascular endothelia, potentially limiting ability for transendothelial migration and metastasis to lung tissue [26]. Additional research has shown tannin extracted from Sumac significantly reduced vascular smooth muscle cell migration [27], hence this may be responsible for the observed anti-metastatic effects in incipient breast cancer cells.

Sumac has also shown anti-angiogenic effects by markedly reducing levels of the vascular endothelial growth factor (VEGF) and PGE2 in MDA-MB-231 cells [26], together fracturing positive feedback regulation between VEGF and PGE2 to suppress angiogenesis [28]. Furthermore, RCE treatment decreased inflammatory cytokines such TNF- α , IL-6 and IL-8 all of which have been implicated in assisting metastasis and angiogenesis within tumour environments, in part, through stimulation of NF κ B pathway [29]. In TNBC, IL-6 and IL-8 autocrine effects are associated with greater tumour growth, resistance to apoptosis and metastasis [30]. RCE decreased levels of TNF- α , and presumably by virtue decreased levels of phosphorylated NF κ B; reduced production of IL-6 and IL-8; and inhibited STAT3 phosphorylation in TNBC cells [26]. Overall, providing substantial evidence that Sumac suppresses metastasis and angiogenesis in TNBC cells.

A Future Therapeutic For Prostate Cancer?

In previous studies, the cytotoxic properties of Sumac extract in the treatment of human breast and colon cancer have been investigated where it inhibited the cell growth, perpetually halted the cell cycle, appeared to induce senescence, apoptosis, autophagy and inhibit metastasis in respective cancer cell line and *in vivo* models [14,24,26]. The downsides of these experiments are the non-use of control cell lines. Taken together, the molecular interrogation and appreciation of Sumac needed to be undertaken in order to assess its further potential role in the molecular and precision medicine approach to cancer treatment. Here, we have used prostate cancer (PrCa) as our model in order to thoroughly dissect the benefits of Sumac in case/control analyses.

Methodology

Human PrCa cell lines RWPE-1, PNT2, DU145 and PC3 were acquired from ATCC. Dried and crushed berries of Sumac were purchased from a culinary supplier, Just Ingredients, (Product code: 11SUMA) <https://www.justingredients.co.uk/Sumach-no-salt.html>. Ethanol extract of Sumac (RCE) was prepared as described by Al Dhaheri Y, *et al.* [31]. Cells were grown in a 96 well flat bottom plate at a density of 10,000 cells per well for 24 hours at 37 °C. Seeding density was optimised to ensure cells remained in log growth phase throughout the assay. 500 mg/mL stock of Sumac ethanol extract was prepared in PBS by dissolving 1 mg of dried red residues in 1 mL of PBS. 2X serial dilution of stock was made in following order 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL, 7.8125 mg/mL, 3.90625 mg/mL, 1.953125 mg/mL, 0.9765625 mg/mL, 0.48828125 mg/mL and 0.2441 mg/mL in PBS, which was further diluted to 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.3125 mg/mL, 0.15625 mg/mL, 0.078125 mg/mL, 0.0390625 mg/mL, 0.01953125 mg/mL, 0.009765625 mg/mL, 0.004 mg/mL in culture media and a final concentration of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.062 mg/mL, 0.031 mg/mL, 0.015 mg/mL, 0.007 mg/mL, 0.003 mg/mL, 0.001 mg/mL and 0.0009 mg/mL respectively was transferred to the cultured cells for 24 hours, 48 hours and 72 hours at 37 °C. A dilution series of 10 mM Docetaxel (in DMSO) (1.6 μ M, 0.533333333 μ M, 0.177777778 μ M, 0.059259259 μ M, 0.019753086 μ M, 0.006584362 μ M, 0.002194787 μ M, 0.000731596 μ M and 0.000243865 μ M) (the same concentrations were used for Vinblastine) in media as a positive control; PBS and DMSO (0.4% in media) was used as negative control. Following, treatment plates were removed from the incubator and cooled at room temperature for 30 minutes. 50 μ l of CellTitre-Glo® (Promega) mixture was added in each well. Plates were covered with aluminium foil and kept on a plate shaker for 20 minutes. Plates were analysed on GloMax® Discover Multimode Microplate Reader. Results were obtained as proportional percentage viability by transforming the comparison of treated and untreated cells as 100% and three independent experiments were performed on three different days to take the average as a final representation of results. Statistical analysis was performed using GraphPad Prism 7.

Results and Discussion

In the present study, well characterised (those that recapitulate, in part, the molecular features of clinical tumours) prostate cancer cell lines, including the normal immortalised epithelial cells, RWPE-1 (derived from normal human peripheral prostate and immortalised with transfection of human papilloma virus) [32], PNT2 cell line (derived from normal prostate epithelial cells transfected with a plasmid containing SV40 genome with a defective replication of origin); brain metastatic (DU145), bone metastatic (PC3) and others cell lines. We selected RWPE-1, PNT2 as normal prostate cell lines and DU145, PC3 as aggressive and metastatic prostate cancer cell lines for our experiments.

Initially, Vinblastine was selected as positive control but it did not show any substantial decrease in cell viability (Figure 2A). Subsequently, we decided to use Docetaxel which is a clinically recognised prostate cancer chemotherapeutic drug used since 1995 [33] and was listed as an essential medicine in health system by WHO (WHO Model List of Essential Medicines (19th List). Figure 2B, shows a significant reduction in DU145 cells in

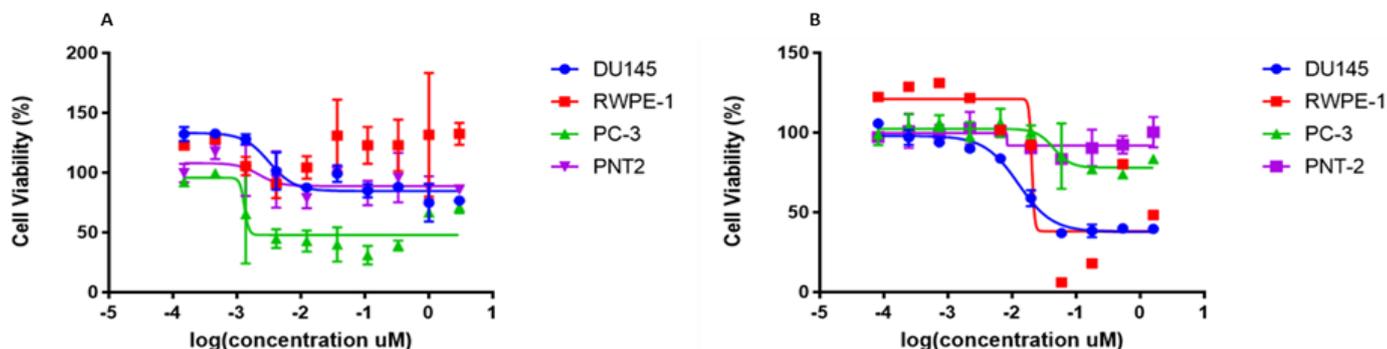


Figure 2. Comparison of prostate cancer cell line viability (%) against Vinblastine and Docetaxel by CellTitre-Glo assay following 72 hours of drug treatment. x-axis represents log of drug concentration in μM and y-axis represents cell viability in percentage (%). Blue and green represents DU145 and PC-3 which are cell line models of aggressive prostate cancer. Red and purple represents RWPE-1 and PNT-2, used as control cell line. Docetaxel has shown a significant reduction in prostate cancer cells growth compared to Vinblastine. Maximum killing effect of Docetaxel was seen against DU145 cells when compared to other cell types with an IC_{50} of $0.012 \mu\text{M}$ which is close to the $0.00808 \mu\text{M}$ IC_{50} <https://www.cancerrxgene.org/translation/Drug/1007>. IC_{50} (μM) were deduced as: 0.003188 , ambiguous, ~ 0.001309 , 0.002219 in the case of Vinblastine and 0.01254 , ~ 0.02037 , $0.04472 \sim 0.008318$ in the case of Docetaxel against DU145, RWPE-1, PC3 and PNT2 cells, respectively.

comparison to other cell types with an IC_{50} of $0.012 \mu\text{M}$ which is experimentally close to the $0.00808 \mu\text{M}$ IC_{50} reported by the Sanger Institute Wellcome Trust in Genomics of Drug Sensitivity in Cancer Research (<https://www.cancerrxgene.org/translation/Drug/1007?>).

All experiments were repeated at 24 hours, 48 hours and 72 hours in 3 independent experiments and a mean of all was taken to report the final results by calculating \log_{10} (RCE concentrations) to analyse the final results on GraphPad Prism 7. A decrease in prostate cancer cell viability was observed when treated with RCE extract, which was more significant than Vinblastine and Docetaxel. The cell viability of cell lines decreased with increased incubation time and the maximum decrease was observed after 72 hours of incubation as shown in Figure 3, IC_{50} of DU145, PC3, RWPE-1 and PNT2 cells lines were 0.1855 mg/mL , 0.1567 mg/mL , 0.08399 mg/mL and 0.0676 mg/mL , respectively. Our results are in agreement with the results reported by [13,24] as they also reported the anticancer activity of RCE against triple negative breast cancer MDA-MB-231 and HT-29 colon cancer cell lines. However, in these studies there was no data offered assessing the RC extract against any normal or control cell line. We further confirmed our results by using the Sulforhodamine B (SRB) cell cytotoxicity assay as described by Orellana EA [34] to validate our findings using an alternative method (results are shown in figure 4).

Conclusion

Cancer is a major health problem affecting millions of people globally. Early diagnosis and intervention have increased chances of survival yet many cancers remain increasingly difficult to treat. Location of tumours, stages of growth, spread and the degree of cellular abnormality present a myriad of challenges. New approaches to treatment are therefore needed to sustain and improve cancer prognosis. Recently, plant derived compounds have been increasingly utilised in the search for potential alternative and complementary therapy. Herbal and plant-based medicines have long been used with significant cultural relevance.

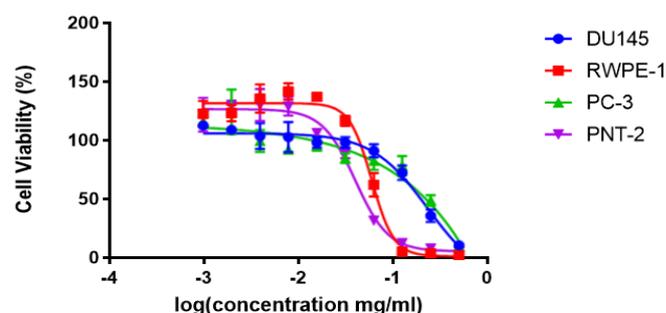


Figure 3. Comparison of different prostate cancer cell line viability (%) against Sumac by CellTitre-Glo assay after 72 hours of treatment. X-axis represents log of *Rhus coriaria* concentration in mg/mL and y-axis represents cell viability in percentage (%). Blue and green colour represents DU145 and PC-3, which are cell lines models of aggressive prostate cancer. Red and purple represents RWPE-1 and PNT2, used as control cell line. Plot has shown increased killing effects of *Rhus coriaria* in comparison to Docetaxel and Vinblastine (Figure 2). However, control cells are equally affected by treatment. IC_{50} of DU145, PC3, RWPE-1 and PNT2 cells lines were 0.1855 mg/mL , 0.1567 mg/mL , 0.08399 mg/mL and 0.0676 mg/mL , respectively.

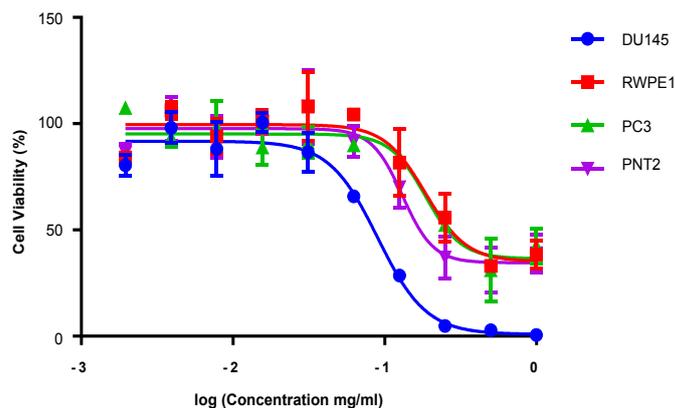


Figure 4. Comparison of different prostate cancer cell line viability (%) against Sumac by Sulforhodamine B assay [33] following 72 hours of treatment. Cells were cultured and tested in the same manner as previously explained yet cell viability was analysed by Sulforhodamine B cell cytotoxicity assay (SRB). IC_{50} (mg/mL) of DU145, PC3, RWPE-1 and PNT2 cells lines were 0.08992 , 0.1856 , 0.1858 and 0.131 , respectively.

The RCE extract discussed here has previously shown promising results in a number of cancer cell line studies, however, none have assessed dose responses in comparison to 'normal' cells. Here we compared RCE to Vinblastine and Docetaxel yet were surprised to observe the extract killed normal cells more aggressively than cancer cells. Thus, obviously active, there are issues with toxicity and the therapeutic index between cancer and normal needs to be explored.

Therapeutic efficacy is widely reliant on the purity and quantity of active components rather than whole fruit extract. There is a need for identification and isolation of the active phytochemicals within Sumac that account for the observed killing effects. This may reduce the stress given to the less dividing prostate epithelial cells immortalised by insertion of viral genome (RWPE1, PNT2) in comparison to fast dividing prostate cancer cells (PC-3, DU145). It will also increase the future prospects of active components of Sumac as an anticancer compound.

Recently, new phenolic compounds Caffeoylquinic acid, Ampelopsin glucoside, Kaempferol rutoside, Rutin and Methyl digallate were identified in RCE [34]. Use of these active compounds together or in combination with other chemotherapeutics may indeed have potentially positive effects to provide an efficacious use of Sumac which in turn may have no effect on normal cells.

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