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A sub-chronic *Xysmalobium undulatum* hepatotoxicity investigation in HepG2/C3A spheroid cultures compared to an *in vivo* model

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ABSTRACT

Ethnopharmacology relevance: Traditional herbal medicines are utilized by 27 million South Africans. *Xysmalobium undulatum* (Uzara) is one of the most widely used traditional medicinal plants in Southern Africa. A false belief in the safety of herbal medicine may result in liver injury. Herb-induced liver injury (HILI) range from asymptomatic elevation of liver enzymes, to cirrhosis and in certain instances even acute liver failure. Various *in vitro* and *in vivo* models are available for the pre-clinical assessment of drug and herbal hepatotoxicity. However, more reliable and readily available *in vitro* models are needed, which are capable of bridging the gap between existing models and real human exposure. Three-dimensional (3D) spheroid cultures offer higher physiological relevance, overcoming many of the shortcomings of traditional two-dimensional cell cultures.

Aims of this study: This study investigated the hepatotoxic and anti-proliferic effects of the crude *X. undulatum* aqueous extract during a sub-chronic study (21 days), in both a 3D HepG2/C3A spheroid model and the Sprague Dawley rat model.

Methods: HepG2/C3A spheroids were treated with a known hepatotoxin, valproic acid, and crude *X. undulatum* aqueous extract for 21 days with continuous evaluation of cell viability and proliferation. This was done by evaluating cell spheroid growth, intracellular adenosine triphosphate (ATP) levels and extracellular adenylate kinase (AK). Sprague Dawley rats were treated with the same compounds over 21 days, with evaluation of *in vivo* toxicity effects on serum chemistry.

Results: The results from the *in vitro* study clearly indicated hepatotoxic effects and possible liver damage following treatment with valproic acid, with associated growth inhibition, loss of cell viability and increased cytotoxicity as indicated by reduced intracellular ATP levels and increased AK levels. These results were supported by the increased *in vivo* levels of AST, ALT and LDH following treatment of the Sprague Dawley rats with valproic acid, indicative of hepatic cellular damage that may result in hepatotoxicity. The *in vitro* 3D spheroid model was also able to predict the potential concentration dependant hepatotoxicity of the crude *X. undulatum* aqueous extract. Similarly, the results obtained from the *in vivo* Sprague Dawley model indicated moderate hepatotoxic potential.

Conclusion: The data from both the 3D spheroid model and the Sprague Dawley model were able to indicate the potential concentration dependant hepatotoxicity of the crude *X. undulatum* aqueous extract. The results obtained from this study also confirmed the ability of the 3D spheroid model to effectively and reliably predict the long-term outcomes of possible hepatotoxicity.

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1. Introduction

Traditional herbal medicines are utilized by a large majority of the South African population. It has been estimated that 27 million South Africans are dependent on traditional herbal medicines provided by a rich diversity of plants consisting of approximately 30 000 plant species (Stafford et al., 2007). *Xysmalobium undulatum* (L.) W.T. Aiton (Apocynaceae), is one of the most widely used traditional medicinal plants of which there are an estimated 1020 in Southern Africa (Stafford et al., 2007; Schmelzer and Gurib-Fakim, 2013). *X. undulatum* is known by a variety of names including; Uzara, milk bush, milkwort (Eng.); melkbos, bitterwortel (Afr.); iyeza elimhlophe, ishongwane (Xhosa); iShinga (Zulu) and is indigenous to sub-Saharan Africa (Kenya, Malawi, Namibia, Angola Botswana, Zimbabwe, Tanzania, Zambia, Lesotho, Mozambique, Swaziland and South Africa) (Makunga et al., 2008; Bester, 2009; Schmelzer and Gurib-Fakim, 2013). Apart from sub-Saharan Africa, *X. undulatum* has also found its way to the pharmaceutical market in Germany (Schulzke et al., 2011). This robust geophyte herb grows approximately 0.5–2.0 m in height, producing almost stalkless large, hairy and heart shaped leaves, with prominent veins and a rounded base. When in bloom during October until December the plant produces cream-green to yellowish flowers with characteristic short white hair growing in small clusters around the stem (Bester, 2009; Schmelzer and Gurib-Fakim, 2013; Vermaak et al., 2004). Large fruits covered with long curly hairs that aid as a “parachute” improving seed dispersion are also present (Bester, 2009; Vermaak et al., 2004). The roots are fleshy, having an almost carrot-like appearance with a nauseating smell (Schmelzer and Gurib-Fakim, 2013).

In traditional remedies the roots and leaves are utilized, however, the stems were found to be poisonous (Reid et al., 2006). Traditional uses of the roots and in some instances the leaves of *X. undulatum* includes the treatment of indigestion and stomach aches, diarrhoea, dysentery, malaria, colic, headaches, sores, wounds and abscesses, afterbirth cramps, dysmenorrhoea, hysteria, cervical cancer, diabetes as well as food poisoning (Steenkamp et al., 2004; Stafford et al., 2005; Reid et al., 2006; Pedersen et al., 2008; Van Wyk, 2011; Schulzke et al., 2011; Schmelzer and Gurib-Fakim, 2013; Vermaak et al., 2004; Kose et al., 2015). *X. undulatum* is traditionally administered as decoctions or teas, for the treatment of internal infections. For other treatments like headaches the plant material is also ground into a powder and administered as a snuff (Schulzke et al., 2011; Kose et al., 2015). Powdered tuber can also be made into a paste by combining it with animal fat and applied as treatment of sores, wounds and abscesses (Van Wyk, 2011; Kose et al., 2015).

Active constituents identified for *X. undulatum* root include the cardenolide cardiac glycosides, uzarin (the main active constituent) and xysmalorin, and their isomers allouzarin and alloxysmalorin. Minor constituents are the cardenolide aglycones, uzarigenin and xysmalogenin as well as, allouzarigenin, alloxysmalogenin, ascleposide, coroglaucigenin, coroglaucigenin-3-*O*-glucoside, pachygenol, pachygenol-3 β -*O*-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β -sitosterol (Vermaak et al., 2004:138). Pharmacologically, Schulzke et al. (2011), found that *X. undulatum* is an effective treatment for acute secretory diarrhoea as it results in the inhibition of active chloride secretions when administered to HT-29/B6 cells at a dosage of 50 μ g/ml. This inhibition results from the inhibition of Na⁺/K⁺-ATPase and a decrease in intracellular cAMP responses and paracellular resistance. Furthermore, in a preliminary clinical study ($n = 46$) by Abd-El-Maeboud et al. (2014), *X. undulatum* was found to be as effective as ibuprofen in the treatment of moderate and severe primary dysmenorrhoea at two initial dosages of 80 mg every 8 h followed by 40 mg every 8 h. However, *X. undulatum* was found not to be pharmacologically active for the treatment of depression when administered orally as single dosages (125, 250 and 500 mg/kg) to c57BL/6J mice subjected to a forced swim test (Pedersen et al., 2008).

Herbal medicines and supplementation are generally believed to be

safe due to their natural origin with fewer adverse effects, however, this is not only misleading but also untrue (Ekor, 2014; Calitz et al., 2015; Boadu and Asase, 2017). Although many of these herbal medicines do offer the potential of becoming valuable candidates in the treatment of various disease states, the majority remain untested and their usage unmonitored while they are introduced as commercial products, without any prior safety or toxicological evaluation (Ekor, 2014). Herb-induced liver injury (HILI) can range from asymptomatic elevation of liver enzymes to cirrhosis, and in certain instances even acute liver failure or hepatocellular carcinoma (Calitz et al., 2015; Wang et al., 2018). Clinical HILI symptoms can be acute or subacute, and these symptoms can range from biochemical liver abnormalities to fatigue, nausea or jaundice, or even no symptoms at all. HILI diagnosis is difficult, but usually involves medical history, physical examination, biochemical assays and imaging. And although liver histopathology can be included, it is not mandatory (Wang et al., 2018).

We have previously reported (Calitz et al., 2018) the incidence of hepatotoxicity and anti-proliferative activity associated with acute administration of a crude *X. undulatum* aqueous extract at concentrations 200, 350, 500 and 750 mg/kg in both two-dimensional (2D) and three-dimensional (3D) cell culture models. Furthermore, there has been reports of a toxic digitalis-like action on the heart when *X. undulatum* is administered in extremely high concentrations (Vermaak et al., 2004).

In an attempt to assess drug biotransformation, hepatotoxicity and HILI, various *in vitro* and *in vivo* models are employed (Tingle and Helsby, 2006; Wrzesinski and Fey, 2015). 2D cell cultures, with cells cultured in suspension or on solid flat surfaces, have been used in drug discovery and screening for decades. But due to poor inter-cellular communication, compromised signalling pathways, the short experimental duration time available and several tissue specific properties lacking, its usefulness as a pre-clinical safety screening model is limited (Lin and Chang, 2008; Breslin & O'Driscoll, 2012; Antoni et al., 2015; Wrzesinski and Fey, 2015; Fang and Eglén, 2017). Therefore, the gold standard for pre-clinical assessment of drug hepatotoxicity remains *in vivo* animal studies. The use of animal models, however, has become the subject of increasing criticism and ethically a strong need exists for the development of novel *in vitro* screening models to effectively reduce the number of animals used in research (Baumans, 2004; Liedtke et al., 2013; Soldatow et al., 2013; Freires et al., 2017). Expression of liver-specific enzymes relevant to drug metabolism and toxicity differ between animals and humans, influencing pre-clinical outcomes (Martigoni et al., 2006; Sabolic et al., 2011; Dalgaard, 2015). Furthermore, *in vivo* animal studies are costly and time-consuming (Soldatow et al., 2013; Sabolic et al., 2011).

Clearly, more reliable and readily available *in vitro* models capable of bridging the gap between the existing models and the *in vivo* environment are needed. This is important since it has been estimated that almost 39% of new drug candidates are withdrawn from development programmes because of pharmacokinetic deficiencies (i.e. inadequacies in absorption, distribution, biotransformation and excretion), while toxicity contributed to withdrawal of a further 21% of drug candidates during the developmental phase (Tingle and Helsby, 2006).

Various advances have been made in the development of novel 3D cell culturing techniques, providing a more reliable system that offers longer-term cell culturing and testing periods and more accurate determination of drug biotransformation and toxicity (Antoni et al., 2015; Brajša et al., 2016; Fang and Eglén, 2017). Of the various 3D models, spheroids have been extensively characterized and offers reproducibility, simplicity and native *in vivo* organ system resemblances (Hirschhaeuser, 2010). Spheroids can be cultured by employing various systems, however Wrzesinski and colleagues have developed a dynamic micro-tissue spheroid cell model capable of overcoming many of the shortcomings of traditional 2D cell cultures (Fey and Wrzesinski, 2012a, 2012b; Wojdyla et al., 2016; Wrzesinski and Fey, 2013; Wrzesinski et al., 2013, 2014; Aucamp et al., 2017).

This study investigated the hepatotoxicity and anti-proliferative effects of

X. undulatum during a sub-chronic 21-day study, both *in vitro* using a 3D dynamic micro-tissue HepG2/C3A spheroid culture model and *in vivo*, using the Sprague Dawley rat model as pre-clinical screening tools. In the 3D model physiological parameters indicative of cytotoxicity and hepatotoxicity was measured, namely intracellular ATP levels, extracellular adenylate kinase levels and spheroid size. In the Sprague Dawley *in vivo* model alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and triglyceride levels were measured as indicators of hepatotoxicity.

2. Materials and methods

2.1. Preparing a crude *Xysmalobium undulatum* aqueous extract

A crude *X. undulatum* aqueous extract was prepared and characterized, and the results previously published (Calitz et al., 2018). Briefly, commercially available dried and milled *X. undulatum* material was purchased from Afrinatural holdings (Prestige laboratory supplies, Mt Edgecombe, Johannesburg). A water extract was prepared by creating an *X. undulatum*-water suspension in a 1:10 product to water ratio. The suspension was sonicated in a Eumax ultrasonic bath at 45 °C for 45 min, followed by centrifugation for 10 min at 5000 × g. The pellet was re-suspended in 100 ml water, and the suspension was sonicated and centrifuged again to collect the supernatant. The collected supernatant was filtered and frozen at –80 °C. The frozen filtrate was lyophilised on a Virtis freeze dryer (SP Scientific, Gardiner, New York).

The chemical composition of the *X. undulatum* aqueous extract was determined on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA), and the presence of the major active constituent, uzarin, was confirmed and quantified (226.4 µg/mg *X. undulatum* (n = 2)), as previously published (Calitz et al., 2018).

2.2. Two-dimensional cell culture

The clonal derivative, HepG2/C3A (American Type Culture Collection [ATCC] cat. no. CRL-10741, sixth passage after receipt from ATCC, Manassas, VA), of the hepatocellular carcinoma cell line HepG2, was cultured using standard tissue culture conditions in Dulbecco's Modified Eagle's Medium (DMEM) (1 g glucose/l) (Gibco, Carlsbad, CA), with 1% non-essential amino acids (Gibco); 10% fetal calf serum (FCS) (Sigma, St Louis, MO); 0.5% penicillin/streptomycin (Gibco) and 1% GlutaMAX (Gibco). Cultures were incubated in a humidified atmosphere at 37 °C, 5% CO₂ and 95% air.

2.3. Three-dimensional cell culture

2.3.1. Spheroid preparation using AggreWell™400 plates

HepG2/C3A cell spheroids were prepared using an AggreWell™400 plate (Stemcell Technologies, Grenoble, France) according to the manufacturers specifications and as previously published (Wrzesinski and Fey, 2013; Aucamp et al., 2017). Wells were rinsed twice with 1 ml AggreWell™ Rinsing solution (Stemcell Technologies, Grenoble, France), and HepG2/C3A cells were seeded into each of the wells of the AggreWell™ plate at a seeding density of 1.2×10^4 . Following centrifugation for 3 min at 120 × g, the plate was incubated overnight to allow spheroid formation.

2.3.2. Spheroid culture in bioreactors

Spheroids were detached from the AggreWell™ wells by gently washing the wells with pre-warmed growth medium. Detached spheroids were then collected, and the spheroid quality was determined using a light microscope. Spheroids were selected based on visual quality, by comparing compactness, size and roundness. Selected spheroids were placed into bioreactors (MC2 Biotek, Hørsholm, Denmark), approximately 300 per bioreactor, and cultivated at 37 °C,

5% CO₂, 95% air in a humidified incubator for 17 days with continuous rotation of the bioreactors.

The day of spheroid removal from the AggreWell™ wells is referred to as day 0 and an estimated 90% of growth medium was removed and replaced on day 3. Thereafter, the medium was exchanged three times per week. The rotation speed of the bioreactors was adjusted as necessary to compensate for spheroid size due to cell growth. The spheroid population was divided on day 8 and when experimental groups were initiated on day 17, and introduced to new bioreactors.

2.3.3. *In vitro* experimental design

Four experimental groups were prepared, and each group consisted of four biological replicates in individual bioreactors. Each experimental bioreactor contained 207 spheroids (17 days old). The experimental groups consisted of spheroids treated with 200 mg/kg (45.28 mg uzarin) and 250 mg/kg (56.60 mg uzarin) crude *X. undulatum* aqueous extract, spheroids treated with 10 g/kg sodium valproate (Sigma, Johannesburg, South Africa) as a positive control for hepatotoxicity, and spheroids in normal growth medium as the untreated control group. Treatment concentrations for *X. undulatum* were based on available literature and previous acute and sub-chronic *in vitro* studies (Vermaak et al., 2004; Calitz et al., 2018), while the valproic acid concentration (10 mg/mg soluble protein, translating to 10 g/kg) was chosen below the acute lethal threshold in spheroids of 20 mg/mg soluble protein, as determined by Fey and Wrzesinski (2013), but high enough to ensure potential hepatotoxic effects would be observable in the model if only limited effects were instigated. To initiate drug treatment, growth medium was exchanged with medium containing the various treatments. Spheroids and medium samples were collected for planimetry, ATP and AK analyses. Growth medium containing treatment was exchanged at 48 h intervals, for a duration of 21 days.

2.3.4. Spheroid microscopy and planimetric analyses

Photomicrographs were taken of three spheroids per time point, in 24 h intervals, for the duration of the 21-day study using an Olympus IX81 motorized microscope and an Olympus DP71 camera. Images were transferred to the Olympus AnalySIS Docu program (Soft Imaging System) and the “shadow” areas of spheroids were measured using the “Fitted Polygon Area” function. This indicated the planar surface area of the spheroids in µm² and allowed for the ATP and AK to be related to spheroid surface area. In a previous study by Wrzesinski and Fey (2013), a correlation was found between the spheroid surface area and the protein content of the spheroids, allowing the normalization of data in terms of protein content. Furthermore, this allowed dosages of *X. undulatum* extract to be administered to the spheroids per mass (mg/kg), which corresponds with dosages administered to animal subjects during *in vivo* studies.

2.3.5. *In vitro* adenosine triphosphate quantification

Cell viability estimation was based on the ability of the spheroids to produce ATP. Spheroids from each experimental group (three replicates consisting of two spheroids per 24 h time point) were collected for the duration of the 21 days, and transferred to white opaque microtitre plates (Nunc, Roskilde, Denmark). Growth medium was removed, and the volume adjusted to 100 µl with Hanks buffered saline solution without Ca²⁺ and Mg²⁺ (Gibco, Carlsbad, CA). The cells were then lysed with 100 µl lysis buffer (CellTiter-Glo luminescent cell viability assay, Promega, Fitchburg, WI) and shaken in the dark for 40 min, whereafter the luminescence was measured in a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) using the following parameters: one kinetic window, 10 measurement cycles with 0.3 s of measurements interval time, 2 s delay per measurement, additional 0.5 s delay per position change (repeated twice for each measured plate). The data was normalized with reference to a standard curve for ATP and the untreated control.

2.3.6. *In vitro* adenylate kinase quantification

AK content was measured in the growth medium (140 µl) of each experimental group to determine cell death in 24 h intervals for the duration of the 21-day study, using the Lonza Toxilight assay kit. Growth medium was placed in microtitre plates in triplicate and diluted with five volumes of AK detection reagent. The plate was then incubated in the dark for 20 min before measuring luminescence using a FluoStar Omega[®] (BMG Labtech). A standard curve was prepared in the same way for each assay plate using a dead-cell standard (4.29×10^6 HepG2/C3A cells per ml, which were lysed in lysis buffer). Gain was adjusted based on the highest standard curve values.

2.4. *In vivo* study

2.4.1. *In vivo* experimental design

The *in vivo* study was approved by the AnimCare ethics committee of the North-West University (NWU-00276-17-S5), prior to initiation of the study. Specific pathogen-free male and female Sprague Dawley rats were obtained from the North-West University vivarium (Potchefstroom, South Africa; South African Veterinary Council registered). Test subjects weighing between 150 and 250 g and aged 7–8 weeks were allowed seven days of acclimatisation to laboratory conditions prior to initiation of the study. Housing was limited to groups of three per cage according to sex, with corncob bedding and an artificial 12-h light cycle in a room with a temperature of 22 ± 2 °C and relative humidity of 55 ± 10 %. Test subjects were fed a conventional laboratory diet with drinking water supplied *ad libitum*. The weight of each test subject was recorded prior to the initiation of the experiment, and bi-weekly thereafter. General clinical observations were made daily, and all test subjects were checked for morbidity and mortality twice daily. All procedures conformed to the North-West University vivarium's laboratory animal care standard operating procedures to ensure that good laboratory practice was adhered to in all instances, according to national and international accepted principles and standards for humane handling of animals.

The test substances consisted of 200 and 250 mg/kg crude *X. undulatum* aqueous extract in water and 300 mg/kg valproic acid in phosphate buffered saline (PBS) as positive hepatotoxicity control, with the control group receiving only PBS. Treatment concentrations for *X. undulatum* were based on available literature and previously acute and sub-chronic *in vitro* studies (Vermaak et al., 2004; Calitz et al., 2018), while the valproic acid concentration was based on its EC₅₀ (485 mg/kg) and LD₅₀ (600 mg/kg) values in rodents (Löscher, 2007; Lee et al., 2008). Fresh stock solutions of the test substances were prepared daily and diluted according to the weight of each test subject prior to administration thereof. The test solutions were administered by means of oral gavage as 21 single dosages at 24 h intervals. Each treatment group consisted of 18 rats (9 male, 9 female). On day 7, 14 and 21, a sample population of 6 rats (3 male, 3 female) from each group were euthanized by means of decapitation, and terminal blood samples were collected for serum chemistry analyses.

2.4.1.1. Adverse events. The initial dosage selected for the valproic acid treatment was 600 mg/kg, based on similar studies in literature (Lee et al., 2008; Zhang et al., 2014). However, during the first 24 h post-administration of the first dosage, severe morbidity could be observed, and some mortalities occurred. Subjects presented with difficulty breathing and lethargy approximately 7 h post-treatment and would succumb in their sleep 9–10 h post-treatment. Some participants were not severely affected but did show a loss of weight of approximately 5% of the initial body weight. Several test subjects presented with rails. An adverse event was immediately reported to the ethics committee of the North-West University, and the study was halted to allow amendment of the study design. Following careful consideration and taking into account the EC₅₀ value (485 mg/kg) and the LD₅₀ value (600 mg/kg) of valproic acid, the treatment was changed to 300 mg/kg valproic acid.

This concentration was estimated to produce measurable changes in liver function, without severe adverse events for the test subjects.

2.4.2. *In vivo* serum chemistry

Terminal blood samples ($n = 6$), were collected at time points 0, 7, 14, and 21 days to analyse markers of liver damage. The following parameters were monitored: triglycerides, total albumin, total bilirubin, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). Serum chemistry was analysed by PathCare Pathologists (Potchefstroom, South Africa). Briefly, samples were centrifuged for 15 min at 4000 rpm in a Biofuge[™] Primo[™] centrifuge (Heraeus Instruments, Thermo Fisher Scientific, Johannesburg, South Africa). Collected serum was then analysed using a Beckman Coulter AU480 Biochemistry analyser (Beckman Coulter, South Africa, Johannesburg).

2.5. Statistical analyses

Data analyses on the *in vitro* data were performed with Graph pad, unpaired *t*-test on the area under the curve was performed to indicate statistically significant differences ($p \leq 0.05$). Data analyses on the *in vivo* data were performed with STATISTICA 13.2 (Statsoft, Tulsa, OK, USA) with which one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed to indicate statistically significant differences ($p \leq 0.05$).

3. Results and discussion

3.1. *In vitro* study

3.1.1. Planimetry and spheroid growth

Planimetric evaluation of spheroid size over time provides a direct indication of spheroid growth and protein content (Wrzesinski and Fey, 2013). The planimetric data, shown in Fig. 1, clearly indicate significant concentration-dependent growth inhibition following treatment with both 200 mg/kg and 250 mg/kg crude *X. undulatum* aqueous extract when compared to the untreated control group. The experimental group treated with 10 g/kg valproic acid displayed even more extensive growth inhibition. The average spheroid surface area at time 0 h for all the experimental groups was $5.38 \pm 0.11 \mu\text{m}^2$, and on day 21 this increased to $17.73 \pm 0.62 \mu\text{m}^2$ for the untreated control, $6.36 \pm 0.36 \mu\text{m}^2$ for valproic acid and $11.23 \pm 0.79 \mu\text{m}^2$ and $11.51 \pm 0.28 \mu\text{m}^2$ for the crude *X. undulatum* aqueous extract at 200 mg/kg and 250 mg/kg, respectively. A statistically significant decrease in the growth curve of the valproic acid compared to the untreated control group ($p < 0.0001$) was observed on day 21, as well as the crude *X. undulatum* aqueous extract at 200 mg/kg and 250 mg/kg relative to the untreated control ($p < 0.0001$). This growth inhibition corresponds well to previous studies with valproic acid and crude *X. undulatum* aqueous extract at various concentrations (Wrzesinski and Fey, 2013; Calitz et al., 2018). The crude *X. undulatum* aqueous extract contains the cardenolide uzarin, which has been shown to possess cytotoxic and anti-proliferative effects (Krishna et al., 2015; Calitz et al., 2018).

3.1.2. Intracellular adenosine triphosphate and extracellular adenylate kinase levels

The intracellular ATP levels indicative of cell viability, expressed relative to the spheroid surface area are depicted in Fig. 2. There was a sharp increase in ATP levels for all the experimental groups within the first 24 h, but then decreased to levels below that of the untreated control group after 48 h exposure. ATP levels for the valproic acid (10 g/kg) positive hepatotoxicity control group remained below that of the untreated control group, decreasing daily from days 5–14. By day 14, the intracellular ATP levels for the valproic acid group was significantly lower than that of the untreated control group ($p < 0.0001$), as well as the crude *X. undulatum* aqueous extract experimental groups.

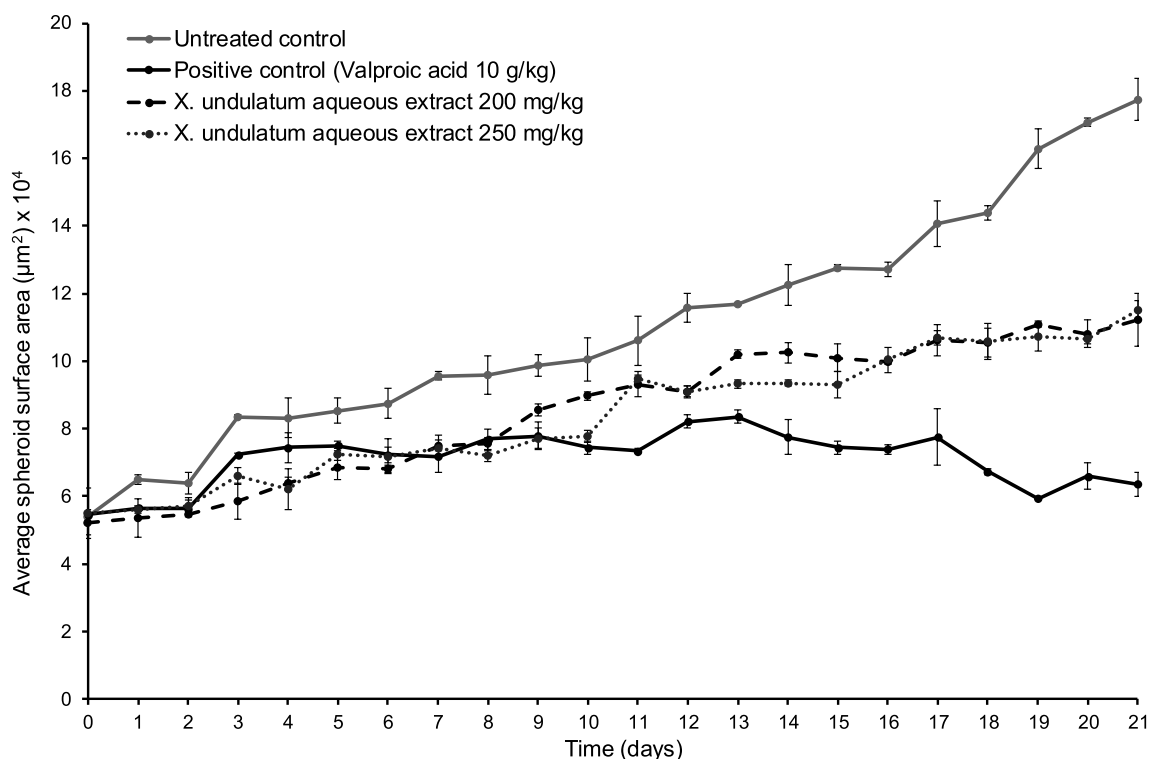


Fig. 1. Average spheroid surface area (µm²) for all experimental groups as a function of time, over a 21-day period (n = 3) (Error bars = standard deviation).

Following treatment with the *X. undulatum* extract, the intracellular ATP levels increased post-treatment, but decreased prior to each successive treatment. By day 19 and 21, ATP levels increased when compared to the untreated control, and this may indicate an attempt by the spheroids to recover from the continuous onslaught of treatment with the crude aqueous extract. This once again corresponds well with

previous data on crude *X. undulatum* aqueous extract (Calitz et al., 2018), although in this study it was observed following extended exposure time.

Fig. 3 shows the extracellular AK released per spheroid surface area as an indicator of active cell death. An inverted trend can be observed between the AK released and the intracellular ATP levels depicted in

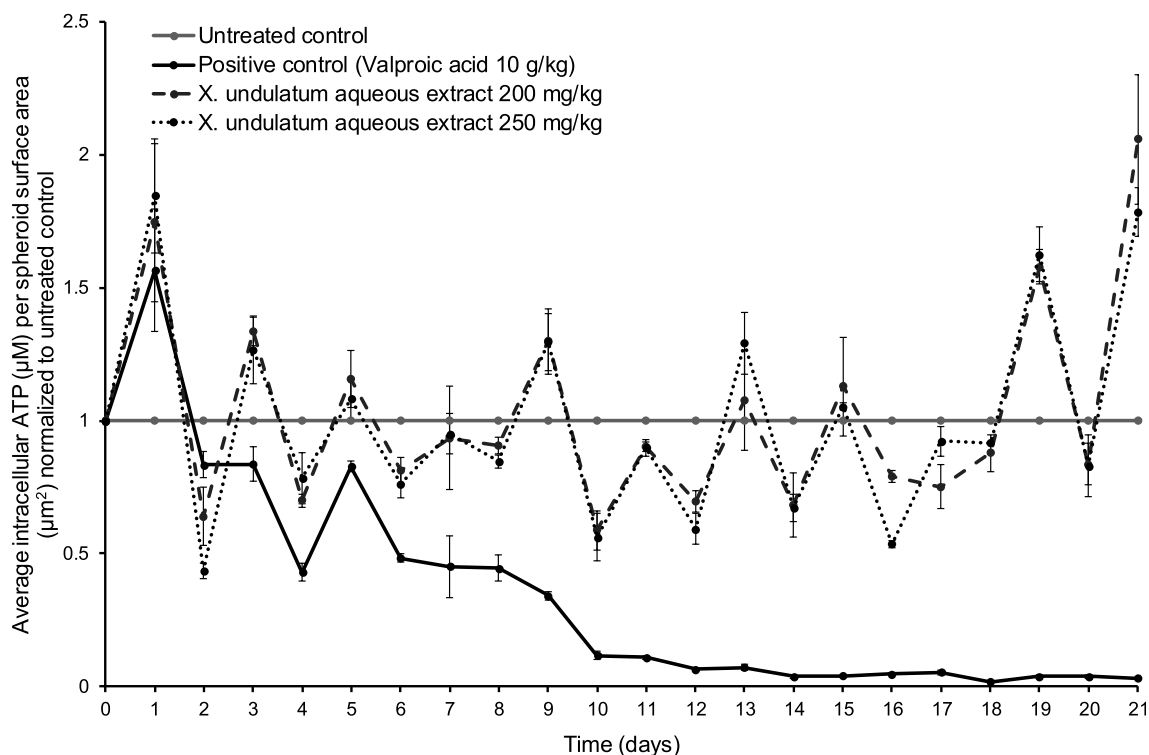


Fig. 2. Average intracellular adenosine triphosphate (ATP) levels (µM) per spheroid surface area (µm²), normalized relative to the untreated control, as a function of time (n = 3) (Error bars = standard deviation).

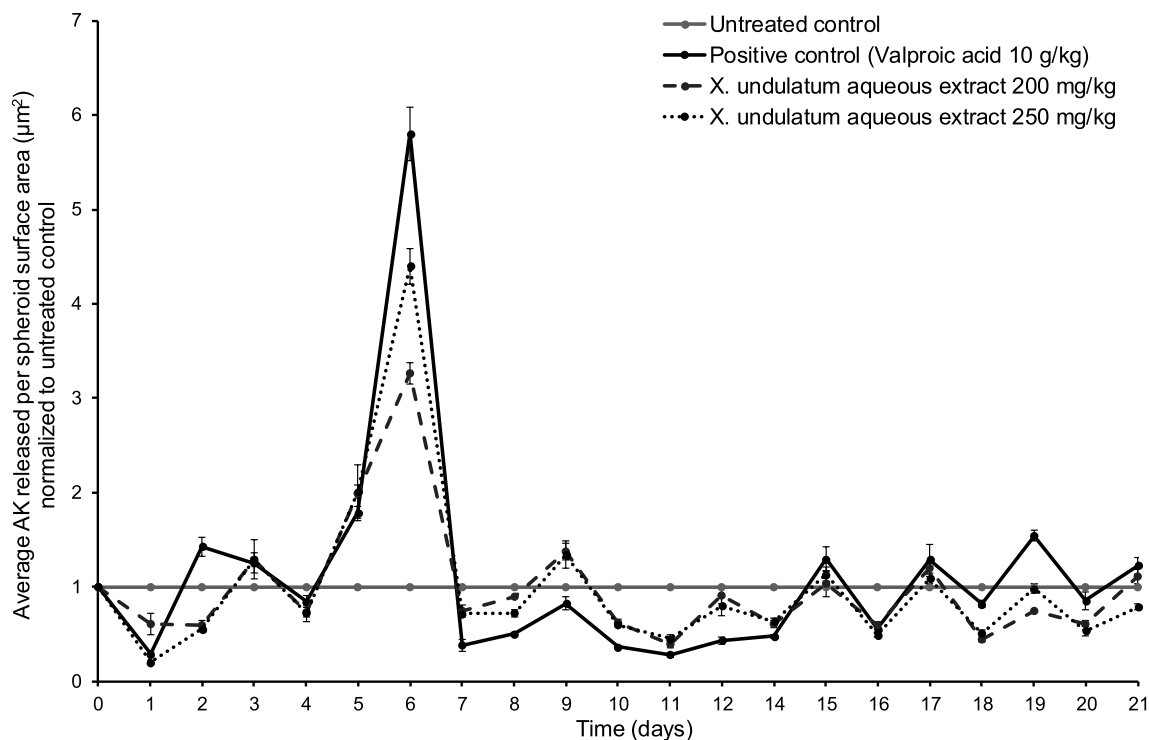


Fig. 3. Average adenylate kinase (AK) released per spheroid surface area (μm^2), normalized relative to the untreated control as a function of time ($n = 3$) (Error bars = standard deviation).

Fig. 2. Normalized AK release appears to have decreased for all experimental groups relative to the untreated control within the first 24 h post-treatment. Extracellular AK then increased to levels above that of the untreated control for the valproic acid treated group, while remaining below the untreated control for the crude extract-treated groups. On day 6, there was an increase in extracellular AK levels for all experimental groups, but by day 7 these levels decreased to lower than that of the untreated control again. Normalized extracellular AK levels remained below that of the untreated control group from day 7 to day 14 for the valproic acid treated group, but on days 15, 17, 19 and 21 the AK levels once again increased above that of the untreated control. The AK released by the *X. undulatum* treated groups mostly remained lower than that of the untreated control for the remaining treatment period.

It is known that an interplay between intracellular ATP levels and the levels of AK released exists and can be indicative of viability. In many instances, hepatotoxicity is associated with the production of reactive oxygen species (ROS) and ultimately with damage to the mitochondria on the organelle level. The latter results in the dysfunction of the mitochondria, determining the fate of the cell, i.e. survival and/or apoptosis/necrosis (Rasda and Bernardi, 2007; Singh et al., 2011). The interplay between intracellular ATP levels and the release of the intermembrane proteins AK and cytochrome *c* (Cyt *c*), can be used to determine mitochondrial dysfunction on the molecular level and ultimately as indicators of cell viability and toxicity. AK release during stressful cellular states is responsible for the up and/or down regulation of intracellular ATP levels, and additionally the release of AK and Cyt *c* activates the execution caspase cascade resulting in apoptosis/necrosis (Single et al., 1998; Leist et al., 1999; Rasda and Bernardi, 2007; Hardie, 2003; Singh et al., 2011).

This interplay can clearly be seen for both the valproic acid hepatotoxicity control and the crude extract treated groups. The increase and subsequent decrease in intracellular ATP levels, and the concurrent increase in AK released by the spheroids treated with valproic acid, is indicative of the known toxicity of this drug and the subsequent loss of cell viability following continuous administration thereof. Similar results were obtained following treatment of the HepG2/C3A spheroids with *X.*

undulatum, albeit to a lesser extent, also indicating toxicity associated with an initial loss of cell viability for both concentrations crude extract. Increased cellular activity at the beginning of the treatment indicates that the treatment compound is not inert. Whether it develops into toxicity, as in did in this case, usually depends on the compound dose. It appears that the HepG2/C3A spheroids actively tried to overcome or repair the damage induced by the crude aqueous extract, but that higher doses and longer treatment led to cellular toxicity.

3.2. In vivo study

Although clinical HILI symptoms are not well known or described (Wang et al., 2018), specific biochemical markers are clinically employed in the diagnosis of liver injuries caused by hepatotoxins like drugs and/or herbal constituents. These liver injury-related biochemical markers include, but are not limited to, albumin, total bilirubin, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and triglycerides (Singh et al., 2011). These markers were therefore quantified in serum samples obtained following treatment of male and female Sprague Dawley rats with valproic acid (300 mg/kg) and crude *X. undulatum* aqueous extract (200 and 250 mg/kg) for 21 days, and are shown in Table 1.

Albumin and total bilirubin levels (data not shown) did not change as a result of the various treatments. Similarly, the ALP levels for all the various experimental groups remained unchanged. Following treatment with 250 mg/kg crude aqueous extract, AST levels increased relative to the untreated control group for the male participants on day 7 and on day 21 for the female participants, while ALT levels were increased at all time points compared to the untreated control group. The valproic acid group also had increased AST and ALT levels after 14 and 21 days' treatment for both male and female test subjects. Increased AST and ALT levels can be indicative of hepatitis, associated with the leakage of these enzymes from damaged mitochondria and cytoplasm (Singh et al., 2011; Featherstone, 2007).

Increased LDH levels are also frequently associated with toxicity,

Table 1

Serum chemistry following sub-chronic treatment of male and female Sprague Dawley rats with valproic acid and crude *X. undulatum* aqueous extract (Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), triglycerides (TG), male (M; n = 3), female (F; n = 3; * Statistically significant values)).

Time point (day)	Parameter	Male/Female	Control ($\bar{x} \pm SD$)	Valproic acid 300 mg/kg ($\bar{x} \pm SD$)	<i>X. undulatum</i> aqueous extract 200 mg/kg ($\bar{x} \pm SD$)	<i>X. undulatum</i> aqueous extract 250 mg/kg ($\bar{x} \pm SD$)
7	ALP (IU/L)	M	757.33 \pm 21.29	427.66 \pm 34.56*	665.33 \pm 77.04**	569.33 \pm 37.57*
		F	597 \pm 76.08	386.33 \pm 49.38*	420.66 \pm 41.33*	538.66 \pm 25.22
	AST (IU/L)	M	247.00 \pm 45.10	203.66 \pm 27.18	222.00 \pm 8.16	305.33 \pm 58.78
		F	299.66 \pm 63.98	213.00 \pm 4.96	271.00 \pm 19.30	290.33 \pm 47.04
	ALT (IU/L)	M	82.66 \pm 7.84	76.66 \pm 3.77	76.00 \pm 4.32	85.33 \pm 7.03
		F	80.00 \pm 4.32	68.00 \pm 4.32	79.66 \pm 5.24	94.00 \pm 3.74**
	LDH (IU/L)	M	861.66 \pm 396.00	695.66 \pm 201.55	797.33 \pm 78.10	1235.33 \pm 349.77
		F	1080.00 \pm 267.49	863.66 \pm 63.54	910.00 \pm 65.67	1086.00 \pm 187.51
	TG (mg/dL)	M	1.15 \pm 0.15	0.93 \pm 0.13	0.87 \pm 0.10	1.29 \pm 0.14
		F	0.76 \pm 0.01	1.17 \pm 0.11	0.70 \pm 0.19	0.87 \pm 0.14
14	ALP (IU/L)	M	646.66 \pm 54.16	454.66 \pm 23.45*	555.66 \pm 4.10*	546.33 \pm 15.19
		F	439 \pm 16.87	307 \pm 22.64	494.66 \pm 52.00	449.33 \pm 33.63
	AST (IU/L)	M	196.66 \pm 31.35	280.00 \pm 19.61	199.00 \pm 28.39	163.66 \pm 4.92**
		F	207.66 \pm 30.72	276.66 \pm 26.7	193.00 \pm 33.17	215.66 \pm 17.55
	ALT (IU/L)	M	71.66 \pm 5.24	93.66 \pm 8.21*	76.00 \pm 4.08	68.00 \pm 1.63**
		F	63.00 \pm 0.81	77.00 \pm 2.44	72.00 \pm 3.55	76.66 \pm 6.64
	LDH (IU/L)	M	654.66 \pm 159.91	975.66 \pm 109.36	653.66 \pm 199.06	542.66 \pm 28.52
		F	588.33 \pm 96.23	989.66 \pm 50.28	645.00 \pm 183.58	705.66 \pm 67.60
	TG (mg/dL)	M	1.01 \pm 0.11	1.14 \pm 0.46	1.14 \pm 0.24	0.96 \pm 0.05
		F	0.64 \pm 0.04	0.79 \pm 0.01	0.78 \pm 0.08	1.53 \pm 1.03
21	ALP (IU/L)	M	584.66 \pm 40.54	568.00 \pm 45.74	542.33 \pm 22.29	491.00 \pm 24.91
		F	444.66 \pm 58.02	379.00 \pm 4.00	456.33 \pm 52.68	379.00 \pm 21.77
	AST (IU/L)	M	213.33 \pm 23.15	288.00 \pm 11.34	212.33 \pm 28.94	185.66 \pm 16.35
		F	197.66 \pm 27.82	376.00 \pm 40.00*	187.66 \pm 25.31**	214.33 \pm 23.80**
	ALT (IU/L)	M	68.00 \pm 4.24	93.00 \pm 4.08*	71.66 \pm 1.24**	70.66 \pm 7.36**
		F	65.00 \pm 7.07	98.00 \pm 4.00*	61.33 \pm 3.09**	78.00 \pm 10.03
	LDH (IU/L)	M	615.33 \pm 114.42	1252.00 \pm 116.01	640.66 \pm 67.59	662.66 \pm 56.38
		F	647.66 \pm 110.97	1708.50 \pm 244.50*	594.33 \pm 78.01**	514.00 \pm 73.33**
	TG (mg/dL)	M	1.51 \pm 0.20	1.14 \pm 0.29	1.42 \pm 0.31	1.52 \pm 0.54
		F	0.96 \pm 0.16	1.08 \pm 0.05	0.81 \pm 0.30	1.31 \pm 0.36

indicating the disruption of the mitochondria and the sarcoplasmic reticulum, and leakage of the enzyme from damaged tissue. Furthermore, it is also indicative of possible hepatocellular necrosis (Singh et al., 2011). Following treatment with the crude *X. undulatum* aqueous extract at 250 mg/kg, the LDH levels increased for both male and female participants on day 7, and for the male participants on day 21. The increased LDH levels were also seen for the male participants of the 200 mg/kg crude *X. undulatum* aqueous extract treated group on day 21 when compared to the control group. The valproic acid treated test subjects also showed a cumulative increase in LDH levels from day 14–21, for both the male and female participants. Furthermore, an increase in TG levels were observed at all time points following treatment with crude 250 mg/kg *X. undulatum* aqueous extract, and also on day 14 for the individuals treated with 200 mg/kg crude *X. undulatum* aqueous extract. The valproic acid treated group showed only a slight increase in TG levels on days 14 and 21. Since the liver is responsible for lipid metabolism, liver dysfunction due to hepatotoxin exposure could also influence lipid metabolism (Featherstone, 2007; Merrel and Cherrington, 2011).

4. Conclusion

Traditional herbal remedies, such as *X. undulatum* have been in use for decades and is still widely accepted as treatment for a variety of ailments. More recently the safety and efficacy thereof has been questioned. This study investigated the possible hepatotoxicity of *X. undulatum* and the ability of more relative and reliable *in vitro* models to predict such toxicity. Due to increasing criticism regarding the use of animal models the scientific community is urged to make a paradigm shift in the means they employ to study incidences drug and herbal toxicity. In this study the results from the *in vitro* study clearly indicated hepatotoxic effects and possible liver damage following treatment with

valproic acid, a compound known to cause hepatotoxic effects in certain individuals, as indicated by the growth inhibition, the loss of cell viability and the increased cytotoxicity as indicated by the reduced intracellular ATP levels and increased AK levels. These results also correlated well with previously published data in both 3D spheroids and *in vivo* systems and is supported by the increased *in vivo* levels of AST, ALT and LDH and the slight increase in triglycerides following treatment of the Sprague Dawley rats with valproic acid, indicative of hepatic cellular damage that may result in hepatotoxicity. The *in vitro* 3D spheroid model was also able to predict the potential concentration dependant hepatotoxicity of the crude *X. undulatum* aqueous extract. Similarly, the results obtained from the *in vivo* Sprague Dawley model indicated moderate hepatotoxic potential.

The results confirmed the usefulness of the HepG2/C3A spheroid culture model to effectively and reliably predict potential hepatotoxicity following long-term exposure to compounds. This model therefore offers a readily available *in vitro* model, capable of bridging the gap between current two-dimensional *in vitro* screening models and the pre-clinical *in vivo* animal models. Implementing this screening model in the pre-clinical evaluation process of new drug entities could therefore reduce costs and time as the use of animal models could be reduced as compounds with potential hepatotoxic properties can be eliminated prior to *in vivo* testing.

Through comparison of the *in vitro* results to those obtained following treatment of an *in vivo* animal model and available literature, the validity and potential of the 3D HepG2/C3A model as a screening tool could be confirmed.

Declaration of interest

The authors declare no conflict of interest.

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