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Evaluation of Four Assays to Determine Cytotoxicity of Selected Crude Medicinal Plant Extracts *In vitro*

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Authors' contributions

This work was carried out in collaboration between both authors. Author WC conducted experimental work and wrote the first draft of the manuscript. Author VS edited the manuscript. Both authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: Cytotoxic assessment of plant extracts is crucial during the pre-clinical screening of medicinal preparations selected for further development. The aim of this study was to compare four assays used to test the cytotoxicity of eight African plant extracts, as well as to determine whether the antioxidant properties of the extracts potentially diminished the reliability of the resazurin conversion assay.
Methodology: HepG2 cells were exposed to hot water or methanol extracts of *Acokanthera oppositifolia, Boophane disticha, Solanum aculeastrum* and *Tabernaemontana elegans* for 72 h. Cell viability was determined using neutral red uptake, sulphorhodamine B staining, MTT and resazurin conversion assays. Phytochemical interference in the resazurin conversion assay was assessed in a cell-free environment and antioxidant activity of the crude extracts was determined using the Trolox Equivalence Antioxidant Capacity assay.
Results: Compared to the other three assays, the resazurin conversion assay failed to detect cytotoxicity, even at the highest concentration tested. The sulphorhodamine B staining assay

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showed the highest reproducibility, and compared well to the neutral red uptake and MTT conversion assays. Although extracts possessed moderate antioxidant activity, this did not contribute to the spontaneous conversion of resazurin in the cell-free environment, inferring that cellular conversion of resazurin was up-regulated by the crude extracts, leading to the false negative result of cytotoxicity.

Conclusion: Due to the potential interference between samples and substrates used in cytotoxicity assays, assessment of the assay's suitability should always be conducted. It is possible that the crude extracts increased resazurin-specific enzyme activity through up-regulation, and as such led to higher conversion. Due to the interference, the resazurin conversion assay should not be used when assessing cytotoxicity of plant extracts. It is recommended that *in vitro* toxicological evaluation be performed using multiple cytotoxicity assay, preferably those based on different mechanistic principles to ensure higher accuracy.

Keywords: Cytotoxicity; phytochemical interference; plant extracts; resazurin.

ACRONYMS

EMEM : Eagle's Minimum Essential Medium
HW : hot water extract
IC_{50} : half-maximal inhibitory concentration
MeOH : methanol extract
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NRU : neutral red uptake
NC : negative control
PC : positive control
RZN : resazurin
SEM : standard error of the mean
SRB : sulphorhodamine B
TEAC : Trolox Equivalence Antioxidant Capacity.

1. INTRODUCTION

Determination of cytotoxicity is required for pre-clinical screening of potential drugs, including plant extracts. Various spectrophotometric or fluorometric assays are available. Spectrophotometric methods include the neutral red uptake (NRU) [1], sulphorhodamine B (SRB) staining [2], and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assays [3]. The resazurin (RZN) conversion assay may be performed either spectrophotometrically or fluorometrically [4]. The NRU and SRB assays are considered inert, and rely on the uptake of dye into viable lysosomes [1] or cellular protein staining [2], respectively. The MTT and RZN assays rely on cellular reduction of the substrate to either formazan (a water-insoluble dye) [3] or resorufin (a highly fluorescent molecule) [4], respectively. Conversion assays, while popular, may be influenced by reductive or antioxidant compounds, which may reduce the substrate spontaneously [5]. This may lead to false positive or negative results [6]. As herbal extracts are complex mixtures of phytochemicals, which includes antioxidants, there is a high potential for inaccurate interpretation of results in these assays.

Various reports on herb-induced liver injury have been published, and pre-clinical, toxicological assessment is an area of concern for herbal drug development. During in-house hepatotoxicity screening of crude extracts using the HepG2 hepatocarcinoma cell line, it was observed that cytotoxicity was underestimated by the RZN assay. Four medicinal plants, namely *Acokanthera oppositifolia* (Lam.) Codd [7] (bark, LT0019), *Boophane disticha* (L.f.) Herb [8]. (bulbs, South African National Botanical Institute), *Solanum aculeastrum* Dunal [9] (fruits, LT0017) and *Tabernaemontana elegans* Stapf [10] (roots, NH1920) are used for the treatment of parasites [7], mental illness [8], cancer [9] and chest infections [10], respectively. However, their effect on hepatocellular cell lines is not well described. The aim of this study was to assess the cytotoxicity of crude extracts of these four plants using the NRU, SRB, MTT and RZN assays, and to determine whether antioxidant activity contributes to the reductive interference in the RZN.

2. MATERIALS AND METHODS

Plant material (10 g) was extracted with either 100 ml methanol (organic extract) or boiling distilled water (ethnomedicinal mimetic extract). Methanol extracts were sonicated for 30 min, shaken for 2 h and incubated at 4°C for 16 h (procedure repeated three times after shaking). Hot water extracts were stirred for 15 min to mimic preparation of a tea. Each extract was filtered (0.22 µm), evaporated to dryness and resuspended to the desired concentrations in Eagle's Modified Essential Medium (EMEM). HepG2 (ATCC #HB-8065) cells (2 x 10^4 cells/well) were exposed to 5% foetal calf serum-supplemented EMEM (negative control) or crude extracts (1, 3.2, 10, 32 and 100 µg/ml) for 72 h. Thereafter, cell viability was determined using the NRU [1], SRB [2], MTT [3] and RZN [4] assays following the methods described by the authors. The interaction between the extracts and RZN was determined in the absence of cells to assess spontaneous conversion. Antioxidant activity of the crude extracts was determined using the Trolox Equivalence Antioxidant Capacity (TEAC) assay [11]. Experiments were conducted in technical and biological triplicate, and GraphPad Prism 5.0 was used for statistical analysis. Results were expressed as the mean ± standard error of the mean (SEM), and the half-maximal inhibitory concentration (IC_{50}) was determined using non-linear regression (normalized variable slope).

3. RESULTS AND DISCUSSION

The RZN assay failed to predict an IC_{50} for the crude extracts, as cell density was >50% at all concentrations tested (Table 1). A hormetic effect was observed in the RZN assay (Fig. 1), where cell viability increased at lower concentrations. This may indicate an adaptive, metabolic response towards the cytotoxicity of the crude extracts. Results differed between the cytotoxicity assays (Table 1 and Fig. 1), especially between the RZN assay and the other three. The SRB and RZN assays offered the least variability between samples.

Table 1. Comparison of cytotoxicity in terms of IC_{50} for the crude extracts using the resazurin, sulphorhodamine B, neutral red and MTT assays

Plant	Extract	IC_{50} (µg/ml) ± SEM			
		RZN	SRB	NRU	MTT
Acokanthera oppositifolia	HW	>100	13.86 ±1.05	9.59 ± 1.07	11.52 ± 1.12
	MeOH	>100	26.63 ± 1.05	30.19 ± 1.00	22.2 ± 1.10
Boophane disticha	HW	>100	69.32 ± 1.08	32.77 ± 1.15	33.16 ± 1.17
	MeOH	>100	45.45 ± 1.07	18.07 ± 1.12	17.17 ± 1.21
Solanum aculeastrum	HW	>100	63.41 ± 1.02	22.02 ± 1.11	34.16 ± 1.10
	MeOH	>100	21.80 ± 1.10	11.28 ± 1.05	15.93 ± 1.06
Tabernaemontana elegans	HW	>100	>100	>100	>100
	MeOH	>100	3.57 ± 1.09	2.86 ± 1.00	4.76 ± 1.13

HW – hot water extract, MeOH – methanol extract

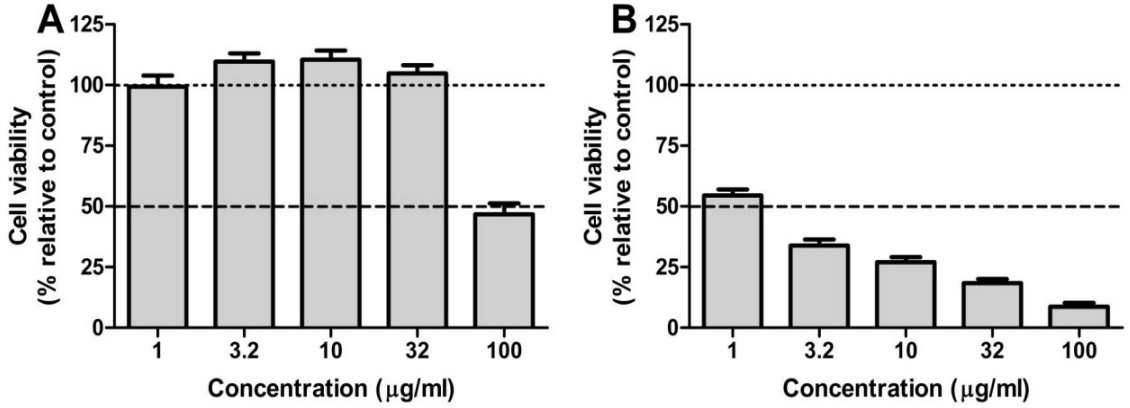

Fig. 1. Comparison of the cytotoxicity of the methanol extract of *T. elegans* as determined by the A) resazurin and B) sulphorhodmine B assays; thin-dashed line represents 100% cell viability, thick-dashed line represents 50% cell viability

The RZN assay under-estimated the cytotoxicity of all four extracts, while the MTT and NRU assays were the most sensitive to cytotoxicity induced by the extracts of *B. disticha* and *S. aculeastrum*. The higher sensitivity of the MTT and NRU assays was also evident from the lower IC_{50} values, which could be indicative of the mechanism of cytotoxicity of these extracts. As the MTT and NRU assays rely on mitochondrial and lysosomal integrity, respectively, it is possible that *B. disticha* may induce mitochondrial dysfunction, while *S. aculeastrum* may antagonize lysosomal stability. Each assay functions on a different end-point for cytotoxicity assessment. Thus these may be antagonized to different degrees and at different stages by the sample. The amount of interference in the RZN assay was not affected by the solvent used for extract preparation.

Limited information is available on the effect of the eight crude extracts on hepatocellular cell lines, making pre-clinical evaluation critical for hepatotoxicity studies. Only one previous study has reported on the hepatocellular cytotoxicity of one of the samples. A methanol root extract of *T. elegans* displayed similar cytotoxicity (IC_{50}: 5.81 µg/ml) in the HepG2 cell line compared to the present study (IC_{50}: 2.86 – 4.76 µg/ml) [12]. From the cytotoxicity data available on different cell lines, it appears that alcoholic root extracts of *T. elegans* inflict non-selective, potent cytotoxicity in THP-1 leukemia cells (<4 µg/ml) [10], lymphocytes (4.52 and 11.77 µg/ml for resting and stimulated lymphocytes, respectively) and normal human dermal fibroblasts (10.91 µg/ml) [12]. The cytotoxicity of *S. aculeastrum* fruits (hot water: 22.02 – 63.41 µg/ml; methanol: 11.28 – 21.87 µg/ml) was found to be similar to that reported in literature, where water extracts (28.4, 27.9 and 48.5 µg/ml) were less cytotoxic than methanol extracts (17.11 17.8 and 41.9 µg/ml) in HeLa, MCF-7 and HT29 cell lines [9]. The methanol extract of *B. disticha* roots displayed slightly lower cytotoxicity (IC_{50}: 23.3 – 27.8 µg/ml) in the SH-SY5Y neuroblastoma cell line [8] than the bulbs used in the present study. The dichloromethane extracts of *A. oppositifolia* roots and stems resulted in total growth inhibition at ≤15 µg/ml in the TK10 renal, UACC62 melanoma and MCF-7 breast carcinoma cell lines [9], which is more potent than the cytotoxicity induced by the bark extracts (IC_{50}: 9.59 – 30.19 µg/ml). In the latter two studies, it is possible that the difference in plant-part or solvent-type may have been responsible for the difference in results.

There was a slight dose-dependent increase in fluorescent activity when crude extracts were exposed to RZN in the cell-free assays, although this was considered to be too low to cause the discrepancy noted (Fig. 2). The reductive capability of the extracts, as determined by antioxidant activity (Table 2), did not show any trend in the level of RZN interference and was thus considered not to be a major determinant in interference. Spontaneous conversion of RZN was thus not considered as the mechanism of interference. As cells are necessary for this effect to occur, it may be inferred that up-regulation of enzymatic processes took place, leading to increased metabolic activity of the cell and conversion of RZN to resorufin. Adaptive responses to cytotoxicity may influence the level of enzyme expression, and therefore higher conversion of the substrate may be observed. This is supported by the hormetic effect observed in the RZN assay.

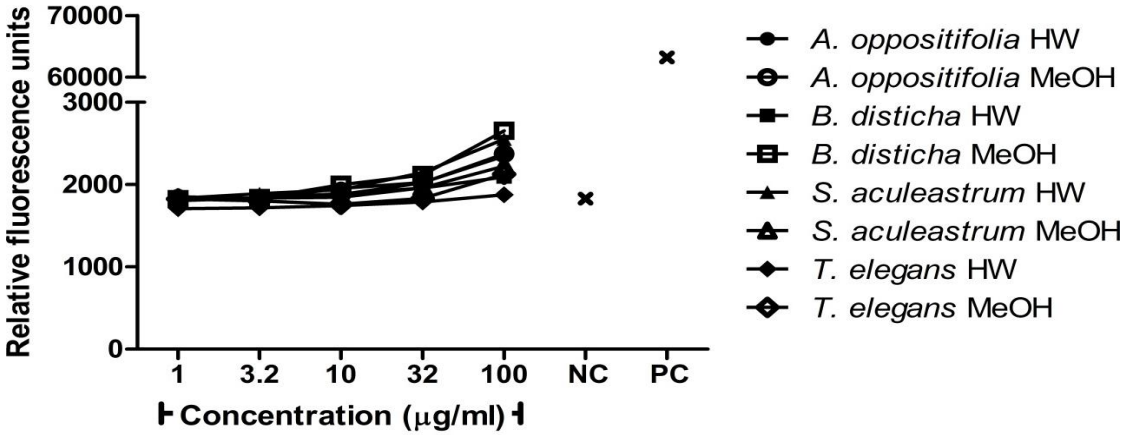

Fig. 2. Cell-free interaction between resazurin and crude extracts; NC – negative control, PC – positive control (1 mg/ml ascorbic acid), HW – hot water extract, MeOH – methanol extract

Table 2. Antioxidant activity of crude extracts in terms of IC_{50} and Trolox Equivalence

Plant	Extract	IC_{50} (µg/ml) ± SEM	Trolox Equivalence (ratio) ± SEM
Acokanthera oppositifolia	HW	16.17 ± 1.03	0.15 ± 0.00
	MeOH	26.39 ± 1.02	0.11 ± 0.00
Boophane disticha	HW	44.54 ± 1.02	0.05 ± 0.00
	MeOH	69.85 ± 1.03	0.04 ± 0.00
Solanum aculeastrum	HW	33.89 ± 1.04	0.09 ± 0.00
	MeOH	19.58 ± 1.03	0.13 ± 0.00
Tabernaemontana elegans	HW	60.68 ± 1.08	0.07 ± 0.01
	MeOH	21.02 ± 1.03	0.13 ± 0.00

HW – hot water extract, MeOH – methanol extract.

As the MTT assay produced similar results to the NRU and SRB assays, the interference appeared to be restricted to RZN conversion. Several mitochondrial and cytoplasmic enzymes result in the conversion of RZN [13], and thus any number of them may act as a substrate for RZN conversion without overlapping with MTT. Glycolysis inhibitors have been shown to interact with MTT, while having no effect on RZN conversion [14]. This may support substrate-specific conversion even though similar enzyme systems are involved. Resorufin is eventually converted to the non-fluorescent dihydroresorufin, but only after a prolonged incubation period [13]. The duration of exposure was thus excluded as a cause of RZN's failure to detect cytotoxicity as plates were only incubated for 2 h and maintained a dark-pink colour. Compounds that have been shown to interfere with the MTT assay, include dicumarol [15], epigallocatechin gallate [6], numerous antioxidants, phytoestrogens and different plant extracts [16]. No literature on isolated phytochemicals or plant extracts interfering with RZN could be found, although it has been shown that redox cycling compounds (that increase reactive oxygen species production) [17] and nanoparticles [18] may influence RZN fluorescence. Reactive oxygen species generation is a common cellular event after exposure to a cytotoxic agent [17], thus it may explain the higher fluorescence observed. Metabolic activity may be affected by the redox state of the cell, which may be influenced by mitochondrial function, reactive oxygen species generation, reduced glutathione levels [15] or the redox potential of the compounds [16]. As this study was conducted in HepG2 cells only, it is possible that cellular type might have an effect on the level of assay interference due to the different mechanisms of toxicity [15].

4. CONCLUSION

All crude extracts, irrespective of solvent type, diminished the activity of the RZN assay, resulting in an underestimation of cytotoxicity in the hepatocellular cell line used. The RZN assay should therefore be avoided in the assessment of herbal-induced cytotoxicity, or should be assessed for suitability prior to experimentation by comparing it to other assays. The SRB assay proved to be the most reproducible, and gave similar results to the the NRU and MTT assays. It is recommended that at least two cytotoxicity assays are conducted to eliminate potential bias and false negative or positive results, thus leading to a higher degree of reliability and a better understanding of the crude extract's potential for cytotoxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERSTS

Both authors have declared that no competing interests exist.

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