

ORIGINAL ARTICLE

Toxicity of *Glandularia selloi* (Spreng.) Tronc. leave extract by MTT and neutral red assays: influence of the test medium procedure

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ABSTRACT

Cytotoxicity assays using cell cultures may be an alternative to assess biological toxicity of plant extracts with potential phytotherapeutic properties. This study compared three methods to prepare culture media for the exposure of Vero cells to plant extracts. Leaves of *Glandularia selloi* (Spreng.) Tronc. were used to prepare culture medium with aqueous extract, extract in culture medium and methanol extract. Toxicity was assessed using the MTT and neutral red (NR) assays. In general, alterations in the cellular functions were found in all extracts and assays. Cytotoxic effect occurred at lower doses in aqueous extract and the range of effect of the methanol extract was small. The procedure of preparing the test medium has an effect on the outcome of the assay. Cytotoxicity of plant extract can be assessed by MTT and NR assays. Aqueous extract added to the culture medium presented the best profile to assess cytotoxicity.

KEY WORDS: cytotoxicity; mitochondrial activity; lysosomal viability; herbal medicine; Vero cell; cell culture medium

Introduction

Traditional or alternative medicine, including herbal medicines, is widely used around the world for both cultural and historical reasons as well as economic factors (World Health Organization, 2013). Phytotherapy has been used in prophylaxis, cure or management of signs and symptoms (World Health Organization, 2005). However, the therapeutic potential of an herb needs to be confronted with the health risks to ensure its safe use.

Safety, efficacy and quality of herbal medicines are the most important challenges of research in traditional medicine. The World Health Organization (WHO) developed technical guidelines and methodologies for research into these products (World Health Organization, 2013), noting however also that some countries do not follow any regulatory requirements for safety assessment of herbal

medicines (World Health Organization, 2005). Moreover, approximately 20,000 herbal products were reported to have limited evidence of safety and efficacy (Bent, 2008).

In Brazil, the safety of herbal medicine is assessed by bibliographic surveys, preclinical or clinical trials or traditional use (Agência Nacional de Vigilância Sanitária, 2010). The recommended preclinical trials concern classical toxicity tests in animals (Agência Nacional de Vigilância Sanitária, 2004). Recently consciousness of ethical issues pertaining to animal experimentation has become more prominent, increasing the need to reduce, replace and refine animal testing (Piersma, 2006). Furthermore, it has also been argued that animal testing is expensive, time-consuming and limited in sample size (U.S. Congress, 1986). Alternative methodologies in biomedical research have lately become the focus of discussion.

From the mid-1980s onwards, cell culture technology began to be extensively used in toxicological research (Zucco *et al.*, 2004). *In vitro* toxicological tests allow the direct or indirect contact of a xenobiotic with the cell culture, thus enabling to assess cell changes. Different mechanisms may be evaluated depending on the assay

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and the cell type used (Rogeró *et al.*, 2003; Freshney, 2005; Khatibisepehr *et al.*, 2011). Some of the most used assays are the neutral red (NR) uptake by lysosomes and the mitochondrial activity test using MTT (Fotakis & Timbrell, 2006). They produce significant data within a short period of time, and that relatively cheaply and with high sensitivity (Rogeró *et al.*, 2003). Both assays are considered reproducible *in vitro* test methods for screening potentially many toxic agents (Mosmann, 1983; National Institutes of Health, 2006). These characteristics suggest that cytotoxicity assays in cell cultures may be used to advantage for assessing the toxicological potential of phytotherapeutic samples (Suares Rocha *et al.*, 2010).

The aim of this study was to evaluate the use of two *in vitro* tests for assessing the cytotoxic effects of plant leave extracts. Moreover, since there are no specific sample preparation protocols for these tests, we evaluated the influence of the recovery vehicle in the performance of the assays.

Methods

Sampling and sample collection

Samples of *Glandularia selloi* (Spreng.) Tronc. (*Verbenaceae*) were collected in the state of Rio Grande do Sul, in Osório, Morro da Borussia, during the flowering period (November). A voucher specimen is deposited in the ICN Herbarium of the Federal University of Rio Grande do Sul (number 190416). *Glandularia selloi* is a creeping plant abundant in Rio Grande do Sul in southern Brazil (Thode & Mentz, 2010). Some native taxa *Verbenaceae* are used for numerous medical purposes, for example *Aloysia gratissima* (Gillies & Hook.) Tronc., *Lippia alba* (Mill.) NEBr. and *Verbena officinalis* L. (Von Poser *et al.*, 1997; Akerreta *et al.*, 2007; Zeni *et al.*, 2011). This stimulated the interest in *Glandularia selloi* (Spreng.) Tronc., which is not used in folk medicine yet has botanical kinship with *V. officinalis* (Thode & Mentz, 2010).

Leaves of the plant were dried in a ventilated place at room temperature and subsequently pulverized in a knife mill. The amount of 0.25 mg of powdered material weighed on an analytical balance was moved to a test tube with a lid after adding 5 mL of methanol. The samples were subjected to ultrasonic extraction for 2 hours without heating. The solution was filtered with filter paper. Afterwards the solution was moved to a 10.0 mL volumetric flask and the volume was adjusted. The filtrate was evaporated to dryness at 40°C in a rotary evaporator.

Preparation of samples and media test

Dry extracts of the leaves were prepared by three different methods.

Method 1 (Aqueous Extract): Water (2mL) was added to the dry extract and filtered using a 0.22 µm filter. The aqueous extract was mixed with Dulbecco's modification of Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS, Cultilab), 10,000 U/mL penicillin and 10 mg/mL streptomycin (Sigma Aldrich)

(standard conditions). The samples were diluted to obtain solutions at 0.1, 0.5, 1, 5 and 10% of the aqueous extract in culture medium.

Method 2 (Extract in Culture Medium): Methanol was added to the dry extract (2 mg/mL). The volume of the extract was reduced by nitrogen evaporation. This dry extract was resuspended in DMEM under standard conditions at the concentration of 2 mg/mL and sterilized by 0.22 µm filter. The samples were diluted to obtain solutions at 0.01, 0.1, 1, 10 and 100 µg/mL of the extract in culture medium and cytotoxicity assays were performed.

Method 3 (Methanol Extract): Methanol was added to dry extract (2 mg/mL). The volume of the extract was dried in water bath at 37°C and redissolved in methanol at the concentration of 2 mg/mL. After sterilization by a 0.22 µm filter, the samples were diluted in DMEM under standard conditions to obtain solutions at 0.01, 0.1, 1, 10 and 100 µg/mL of the extract in culture medium. A methanol control was performed with the solution at 5% in the culture medium.

Cell culture and treatment

Vero cells were kindly donated by Ph.D. Fernando Rosado Spilki (FEEVALE, Brazil). These cells are derived from kidney epithelial cells of a normal adult African Green Monkey.

The cells were grown in DMEM in standard conditions. Culture was maintained at 37°C in a humid atmosphere at 5% of CO₂ and a half-open system, and trypsinization was used for cell maintenance.

For exposure to extracts, the cells were seeded at a rate of 1.5 x 10⁴ cells/well in a 96-well polystyrene microplate. After the cultures had reached 50% of confluence, the culture medium was replaced by the media prepared using one of the three methods at different concentrations and the cells were exposed for 24 hours. Negative control was done with DMEM under standard conditions. All assays were performed in quadruplicates.

Cytotoxicity assay using MTT reduction

In order to evaluate cytotoxicity using the mitochondrial activity parameter, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used as described by Mosmann (1983). After exposure for 24 hours, the assay was performed according to Trintinaglia *et al.* (2015). Briefly, 20 µL of MTT 1.67 mg/mL (Sigma Aldrich) was added to the well and the plates were incubated for two hours. After incubation, the medium was removed by inversion and 200 µL of dimethyl sulfoxide (DMSO) was added to each well for solubilization. All samples were transferred to a 96-well plate and absorbance was measured at 570 nm with a microplate spectrophotometer.

For the purpose of testing the reaction of extract per se, an additional test was carried out. Before MTT incubation, the culture medium with aqueous extract was removed and 200µL of DMEM without serum was added to each well. The assay was performed immediately as described above.

Cytotoxicity assay by neutral red incorporation

The neutral red (NR) incorporation method, described by Borenfreund and Puerner (1985), was used to evaluate cytotoxicity through lysosome viability. After exposure for 24 hours, the assay was performed according to Trintinaglia et al. (2015). Briefly, the culture medium was removed and 200 μ L of DMEM without serum and containing 50 μ g/mL of NR (Sigma Aldrich) was added to each well. After three hours of incubation, the medium was removed and the cells were washed with buffered saline solution (PBS), followed by another washing with a fixative solution (1% CaCl_2 in 0.5% formaldehyde). After discarding by inversion, 200 μ L of 1% acetic acid in 50% ethanol was added for the solubilization of the dye. The samples were gently shaken for 10 minutes for full dissolution. All samples were transferred to a 96-well plate and the absorbance was measured at 540 nm with a microplate spectrophotometer.

Statistical analysis

Data obtained from *in vitro* cytotoxicity assays were expressed as mean and standard deviation. One-way ANOVA and the Duncan or Scheffe post-test were used for statistical analysis with the IBM SPSS Statistics 19.0 for Windows. To assess the interference of the extract in MTT assay, two-way ANOVA was performed. All results were evaluated using a significance level of $p < 0.05$.

Results

The results of cytotoxicity assays are presented in Figure 1. Alterations in the cellular functions were found in all extracts and assays. Cytotoxic effect occurred at lower doses in aqueous extract, i.e. the culture medium with extract resulted in mitochondrial activity or lysosomal

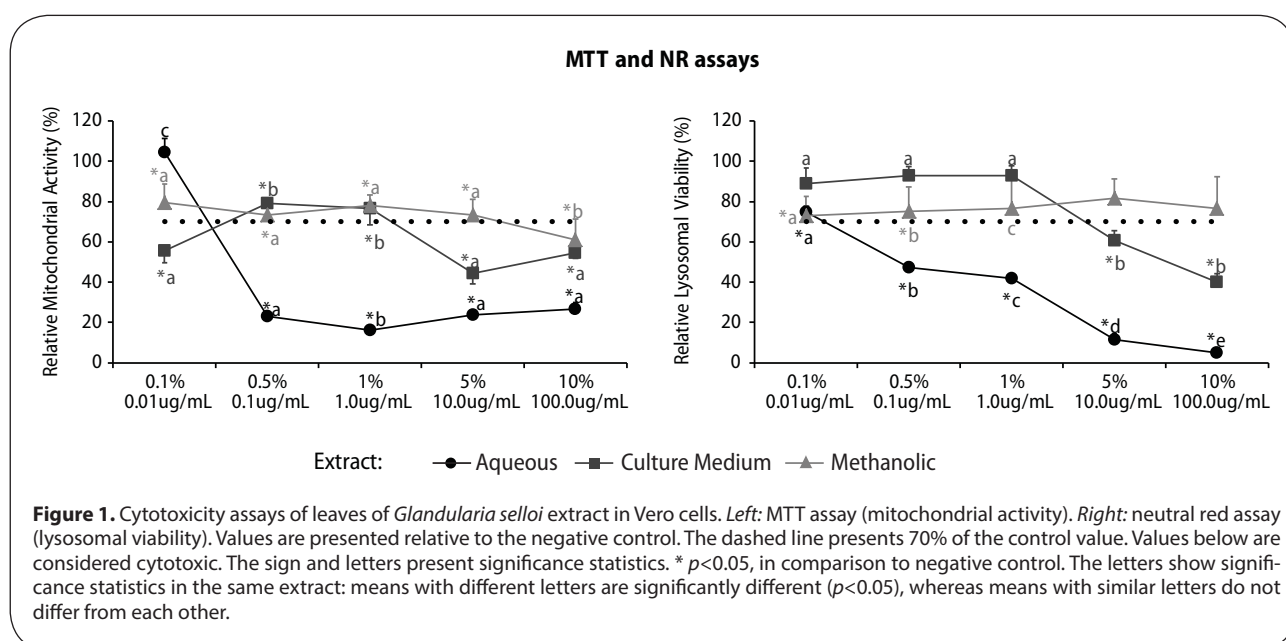
viability below 70% of negative control. The methanol extract had the slightest range of effect. The methanol control was equal to the negative control ($p = 0.989$ to MTT and $p = 1.000$ to NR). Exchange of the culture medium before the incubation in the MTT assay yielded no difference from the usual procedure ($F_{\text{interaction}} = 1.60$; $p = 0.199$) (Figure 2).

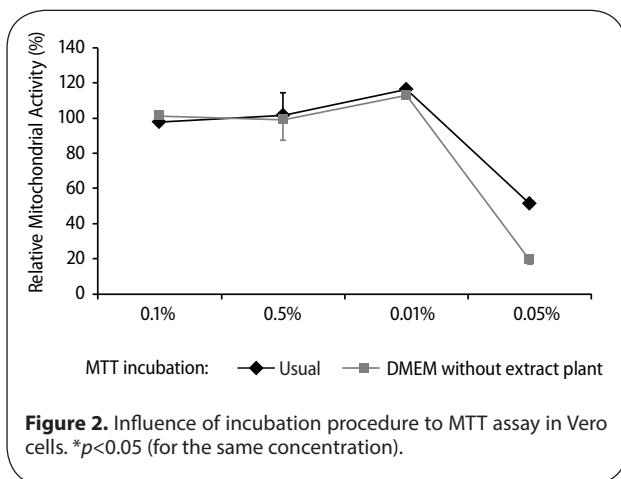
Discussion

Since the MTT assay has been widely used as a screening test to evaluate the cytotoxicity *in vitro*, its applicability in assessing toxicity in plant extracts has been promising. In this study, the MTT and NR assays were able to show cellular effects of the plant extract. Moreover, the aqueous extract seemed to have the most characteristic cytotoxic profile.

The MTT assay is a rapid, economic, sensitive and accurate test to determine *in vitro* deleterious effects of several substances. It shows the disruption of a critical biochemical function and effects of early cytotoxicity (Malich *et al.*, 1997). The test is based on the technique developed by Mosmann (1983), in which a yellow tetrazole water soluble salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced in the inner mitochondrial membrane to a purple formazan salt by the action of succinate dehydrogenase. The resulting product indicates the mitochondrial activity status and reflects the number of viable cells (Triglia *et al.*, 1991; Butler, 2004; Freshney, 2005, Vanden Berghe *et al.*, 2013).

However, formazan is produced by activated cells even in the absence of proliferation (Mosmann, 1983). Moreover, in dead cells, the mitochondria may remain with intact succinate dehydrogenase activity, which is misinterpreted as survival. The cytoplasm, endosome/lysosome compartment and the plasma membrane may





be associated with MTT reduction (Berridge *et al.*, 2005). Some compounds may also enhance or reduce succinate dehydrogenase activity without affecting survival (Vanden Berghe *et al.*, 2013) or still interact per se with the salt MTT (Shoemaker *et al.*, 2004, Peng *et al.*, 2005; Lupu & Popescu, 2013). The latter is particularly important since many plant extracts have compounds with intrinsic reductive potential, such as thiols or other antioxidants (Bruggisser *et al.*, 2002, Shoemaker *et al.*, 2004). This does not seem to occur with the aqueous extract since the effect of different concentrations did not depend on the procedure used in MTT assay as there was no interaction between them. This indicates that it is not necessary to change the medium before incubation.

A further assay that provides a quantitative estimation of the number of viable cells is the NR assay, described by Borenfreund and Puerner in 1985. It is based on the capacity of a vital soluble dye to cross the plasmatic membrane and be incorporated in lysosomes of viable cells (Borenfreund *et al.*, 1988; Triglia *et al.*, 1991). In normal conditions, the lysosome presents a pH lower than that of the cytoplasm. The dye trapped by the lysosome becomes charged and is unable to leave the interior of the organelle. Damaged lysosomes decrease the amount of dye retained by the culture. Thus lysosomal integrity is a highly sensitive indicator of cell viability (Repetto *et al.*, 2008). Since its introduction, this assay has proved technically fast, simple, cheap, sensitive and reproducible (Zhang *et al.*, 1990). The lysosome is the terminal organelle of the endocytic pathway, thus the NR assay is considered to be the measurement endpoint (National Institutes of Health, 2001; Česen *et al.*, 2012).

Both assays use colorimetric measurements to define the number of viable cells after exposure to xenobiotics. However, different cytotoxicity assays may yield different results as each assay determines a different function (Borenfreund *et al.*, 1988, Kim *et al.*, 2009) and the absence of mitochondrial or lysosomal damage does not indicate nonexistence of cellular injury. Studies with different substances found that NR and MTT assays possessed a higher sensitivity for the detection of toxicity than did other assays, as e.g. lactate dehydrogenase and

protein content assays (Arechabala *et al.*, 1999; Fotakis & Timbrell, 2006). Thus these two checkpoints have been commonly used with a reasonable degree of success since they are based on cellular events that have indirect if not direct relevance to cellular responses to chemicals in vivo (National Institutes of Health, 2001). On the other hand, the effects may be different depending on the exposure time. One of the problems in evaluating toxicity is the choice of exposure time influencing the targets to be monitored (Zucco *et al.*, 2004). Lack of an adverse cellular event does not indicate absence of damage since only one timepoint was analyzed and the kinetics of the cell death process were not established.

The plant extracts caused alterations in the two assays studied, suggesting that there were substances in these extracts that interfered with cell function. In general, the two tests showed similar results. The dose-response relationship in the MTT assay was not always established as higher concentrations showed in some instances an increased functionality than did lower concentrations. The aim of this work was not to investigate substances in *Glandularia selloi* responsible for the cellular effect, however leaves of herbal medicine may be rich in flavonoids and other substances with protective effect (Bent, 2008) that consequently increase cellular response in more concentrated solutions. A study for establishing this possibility is being prepared.

The results differed according to the method of preparation of the extract. It is a critical stage because the extract should correspond to the content of the crude product. Different biologically active compounds may be released according to the solvent used due to the physico-chemical properties of the substances and the interaction between them. This can have a direct impact on cellular outcomes and it may be interesting to run experiments with different sample preparation in order to identify different effect profiles. Methanol extract was the one that showed the lowest range of effect. As it was the one to be heated, it may be assumed that temperature influenced the activity of cytotoxic substances. This hypothesis needs to be proven in further studies. Moreover, it is important to consider the effect of the recovery vehicle, which may be downward and mask a possible dose-dependent effect. It does not seem to be the case in this study since the methanol at 5% (the highest concentration in the dilutions) was equal to the negative control.

Compounds need to be chemically characterized and investigated for their pharmacological and toxicological properties. As herbal medicines are highly complex mixtures of potentially active ingredients, the isolated natural compounds and the final product should undergo the same tests (Bunel *et al.*, 2014).

Conclusion

Our study showed that MTT and NR assays can be valuable tools for determining the cytotoxicity of leaves of *Glandularia selloi* under controlled *in vitro* conditions.

However, some factors may affect the outcome of the assays. We found that aqueous extract added to the culture medium presented the best profile for assessing cytotoxicity. The extract reduced by nitrogen evaporation and recovery in culture medium provides a better choice compared to work with methanol extract.

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Declaration of interest statement. The authors report no conflict of interest.

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