In vitro methodological standardization of MTT colorimetric assay for evaluation of formazan activity and drug dosage

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Abstract

Background

Aiming to develop essential chemotherapeutic drugs to control various types of cancer, one of the primary tests is the tetrazolium reduction method. The present study aims to standardize the electromagnetic spectrum that best discriminates the absorbance and to evaluate the drug dosage of single and daily doses during the cell viability and toxicity analysis by the tetrazolium reduction method, using the ethanol extract of pequi peel in canine osteosarcoma cells.

Results

The wavelength of 532 nm showed the best results. We found that as the treatment time increased, the formazan conversion reduced. After 72 hours of treatment, we observed clear discrimination of dose-dependent data, with up to five discriminants within 72 hours with a change in absorbance from 0.554 to 0.064 A. The wavelength of 570 nm it was not ideal since we could not discern the difference in the spectral reflectance of the treatments and, therefore, show no statistical difference among treatments. We found no statistical difference for the coefficient of variation at wavelengths of 532 and 570nm, which were 12.77% and 8.80% respectively.

Conclusions

The wavelength of 532nm best discriminated the absorbance, as it presented better ability to congregate the treatment groups, greater variation between the discriminant and lower coefficient of variation, during the colorimetric test to evaluate the cellular metabolism. Moreover, the ethanol extract of pequi peel in canine osteosarcoma cells showed a statistically equal effect of a single dose administration to the dose reapplied every 24 hours.

Background

The use of experimental models in comparative research aims to generate data that can clarify or extrapolate responses to human medicine, aggregate information at molecular levels of neoplasms, add pharmacological interaction, show effective treatment protocols and possible side or adverse effects. Besides, they bring into question the true pharmacological applicability in human tumors.

The use of cultured cancer cell lineages for therapeutic purposes generates information of their etiology and treatment of diseases of carcinogenic origin, spontaneous or not induced [1].

Aiming to develop essential chemotherapeutic drugs to control various types of cancer, one of the primary tests is the tetrazolium reduction method. This test can determine the minimum inhibitory concentration required to obtain 50% of viable or unviable cells by the cytotoxicity promoted by the tested agent, the time to drug exposure and the efficacy of drug administration [2, 3].

This test is based on the quantitative analysis of dehydrogenase activity of NADH or NADPH reaction products, which in turn reduces MTT [3-(4,5- dimethyl − 2- thiazolyl) -2,5-diphenyl-2H- tetrazolium] formazan activity in viable cells. Formazan crystals of intense purple-blue color are quantified by spectrophotometer. Under properly optimized conditions, the absorbance value obtained is directly proportional to the number of living cells [4].

However, one of the main obstacles found during an experiment is stablishing and standardizing each technique to be used. The inaccuracy of the methodological technique adopted enables mistakes in the resolution and interpretation of the obtained results [5].

The present study aims to standardize the electromagnetic spectrum that best discriminates the absorbance and to evaluate the drug dosage of single and daily doses during the cell viability and toxicity analysis by the tetrazolium reduction method, using the ethanol extract of pequi peel in canine osteosarcoma cells.

Results

The wavelength of 532 nm showed the best results. We found that as the treatment time increased, the formazan conversion reduced. After 72 hours of treatment, we observed clear discrimination of dose-dependent data, with up to five discriminants within 72 hours with a change in absorbance from 0.554 to 0.064 A (Table 1).

Table 1
– Tukey's test results of absorbance means from spectrophotometry in the wavelength of 532 nm, after exposure to ethanol extract of pequi peel, at concentrations of 1, 10, 100, 1000 µg/mL and control, during 24, 48 and 72 hours.

Time	Treatment	Mean	Tukey
24 hours			
	1 µg/mL	0,554	a
	100 µg/mL	0,548	ab
	10 µg/mL	0,524	ab
	CON1	0,519	ab
	CON2	0,484	b
	1000 µg/mL	0,136	c
48 hours			
	CON1	0,495	a
	1 µg/mL	0,490	a
	CON2	0,468	a
	10 µg/mL	0,359	b
	100 µg/mL	0,279	c
	1000 µg/mL	0,083	d
72 hours			
	CON1	0,582	a
	CON2	0,493	b
	1 µg/mL	0,420	c
	10 µg/mL	0,312	d
	100 µg/mL	0,289	d
	1000 µg/mL	0,064	e
*CON1: control without DMEM; CON2: control with DMSO.			

Similar results can be observed in the 48-hour group, in which control 1, control 2 and 1 µg/mL treatment showed no statistically significant difference, as the lower extract concentration (1 µg/mL) promoted formazan conversion equivalent to the control analyzed. Besides, the 10 µg/mL, 100 µg/mL and 1000 µg/mL treatments

converted less formazan as the extract concentration increases, in which we found a dose-dependent reduction in MTT conversion.

The wavelength of 540 nm did not show analytical accuracy of results regarding the discriminants of 24, 48 and 72 hours, which begins to show inaccuracy in the quantification or even excitation of formazan by the wavelength of 540 nm that varied from 0.198 to 0.042 A (Table 2).

Table 2
– Tukey's test results of absorbance means from spectrophotometry in the wavelength of 540 nm, after exposure to ethanol extract of pequi peel, at concentrations of 1, 10, 100, 1000 µg/mL and control, during 24, 48 and 72 hours.

Time	Treatment	Mean	Tukey
24 hours			
	1 µg/mL	0,198	a
	100 µg/mL	0,163	ab
	CON1	0,162	ab
	CON2	0,128	b
	10 µg/mL	0,127	b
	1000 µg/mL	0,044	c
48 hours			
	CON1	0,123	a
	CON2	0,115	a
	1 µg/mL	0,099	ab
	10 µg/mL	0,056	ab
	1000 µg/mL	0,044	b
	100 µg/mL	0,041	b
72 hours			
	CON1	0,168	a
	CON2	0,135	a
	1 µg/mL	0,118	ab
	10 µg/mL	0,061	bc
	100 µg/mL	0,044	c
	1000 µg/mL	0,042	c
*CON1: control without DMEM; CON2: control with DMSO.			

The wavelength of 570 nm is the most used absorbance in the literature, however, in the present study, it was not ideal since we could not discern the difference in the spectral reflectance of the treatments and, therefore, show no statistical difference among treatments (Table 3).

Table 3
– Tukey's test results of absorbance means from spectrophotometry in the wavelength of 570 nm, after exposure to ethanol extract of pequi peel, at concentrations of 1, 10, 100, 1000 µg/mL and control, during 24, 48 and 72 hours.

Time	Treatment	Mean	Tukey
24 hours			
	10 µg/mL	0,104	a
	CON1	0,095	a
	CON2	0,095	a
	1 µg/mL	0,095	a
	100 µg/mL	0,090	a
	1000 µg/mL	0,073	a
48 hours			
	1 µg/mL	0,109	a
	CON2	0,107	a
	CON1	0,101	a
	10 µg/mL	0,091	a
	100 µg/mL	0,080	a
	1000 µg/mL	0,062	a
72 hours			
	CON1	0,104	a
	CON2	0,101	a
	1 µg/mL	0,095	a
	100 µg/mL	0,085	a
	10 µg/mL	0,084	a
	1000 µg/mL	0,057	a
*CON1: control without DMEM; CON2: control with DMSO.			

We found no statistical difference for the coefficient of variation at wavelengths of 532 and 570 nm, which were 12.77% and 8.80% respectively. They showed a low variation index, which demonstrates experimental precision. However, the wavelength of 540 nm showed a mean coefficient of variation of 20.80%, i.e., the highest index of variation, revealing the existence of variance in reading or interference of other agents for this wavelength (Table 4).

Table 4
– Comparison of the overall mean percentages obtained from the ANOVA for discrimination of the wavelengths of 532, 540 and 570 nm by Tukey's test.

Spectrum (nm)	Mean (%)	Tukey
540	20.80	a
532	12.77	b
570	8.80	b

We developed a second independent experiment due to the need to evaluate the effect of continuous treatment and treatment reapplication (SID) every 24 hours. Among the results, we found no statistical difference for the two application techniques of the extract (drug), which suggests that applying the extract or reapplying every 24 hours has the same effect for the wavelength of 532 nm (Table 5).

Table 5
– Mean absorbance generated by spectrophotometry in wavelength of 532 nm for single-dose and SID treatments, after exposure to ethanol extract of pequi peel, at concentrations of 1, 10, 100, 1000 µg/mL and control, during 24, 48 and 72 hours.

	24 hours				48 hours				72 hours			
	C		D		C		D		C		D	
CON1	0.34	a	0.30	a	0.36	a	0.29	a	0.39	a	0.35	a
1 MG/ML	0.37	a	0.31	a	0.30	a	0.27	a	0.24	a	0.17	a
10 MG/ML	0.34	a	0.34	a	0.22	a	0.20	a	0.19	a	0.18	a
100 MG/ML	0.32	a	0.26	a	0.19	a	0.16	a	0.13	a	0.11	a
1000 MG/ML	0.25	a	0.18	a	0.23	b	0.04	a	0.08	a	0.06	a

* C: single dose treatment; D: treatment with repetition (every 24 hours – SID); CON1: control with DMEM.

Discussion

In the present study, the ideal wave spectrum standardization was established to better discriminate the absorbance values obtained using the spectrophotometer.

Stockert et al. [6] showed the peak of formazan conversion while working with sunflower oil, demonstrating that the wave spectrum for formazan reading consists of 512 to 587mn with 562mn plateau. They also reported that the spectrum is within the accepted parameters for mineral oil, suggesting that the dilution driver and other factors may influence the physical properties of the samples and thus directly interfere with the ideal wavelength.

Carmichael et al. [7] proved that the plateau for reading the diluted formazan in DMSO was 503 nm and could vary with the concentration of metal ions in the solution.

Rekha and Anila [8] showed the in vitro cytotoxicity of Triethanolamine-coated CaS nanoparticles in human fibroblast cell lines by the MTT assay. In their study, cell viability was greater than 90% at concentrations ranging from 6.25 to 100 µg/mL. However, the mean absorbance of the samples ranged from 1.2667 to 1.1820 A. Values higher than those found in the present study for the same wavelength of 540 nm and also higher than those obtained at the wavelength of 532 nm. This factor demonstrates the occurrence of photocatalytic interactions between nanoparticles and the MTT cytotoxicity indicator, which proves the interaction of other compounds in the analyzed wavelength and justifies the absorbance values found in the study.

The coefficient of variation was a decisive criterion in choosing the best wave spectrum because it evaluates the dispersion in relative terms and how homogeneous the data are. Importantly, biological data have a high coefficient of variation, even though the experimental set was performed in a laboratory environment with controlled variables.

The study proposed by Young et al. [9] showed a coefficient of variation higher than this study, reaching values of 14.8% for the ideal wavelength of 532 nm. Despite the higher values, it showed accuracy and sensitivity in the dose-response effect of the drug. This study reinforces the criterion of choosing the 532 nm wave spectrum as ideal for the present study.

The ethanol extract of pequi peel demonstrated stability and functionality over the treatment time, suggesting a synergism and balance between micro- and macromolecules. Plant extracts can oxidize over time and often make complexes of proteins with macromolecules present in the extract itself, which inactivate their effect, promoting unwanted metabolic activities, or even losing their original effect.

Other researchers have shown a dose-dependent effect over time, as proposed by Vieira et al. [10], which demonstrated apoptosis in cell line U937 and THP-1 by the extract of Croton urucurana in a single application dose. They showed that at concentrations of 400 and 800 µg/mL, and over a period of 48 hours, the extract promotes 75% of apoptosis, demonstrating the desired effect, over a long period, from the ethanol extract.

By extrapolating the results obtained with the possibility of in vivo use, these data become even more promising when referring to the hypothesis of a decrease in multiple drug applications in patients. This could significantly reduce the stress caused by intravenous applications, the transport of patients to treatment locations, or even the time spent for drug administration, which in many cases may take hours.

During the execution of an experiment, all available knowledge in the literature is used to improve the scientific conduct of innovative essays. In this sense, the experimental execution of technique and drug efficiency should

corroborate. The standardization of the cell viability technique contributes to the accuracy of results, as there are factors that influence the expected result. Among those factors, we can emphasize the maturation time and permanence of MTT until formazan conversion, wavelength and cells used, density, osmolarity, accelerated cancer cell metabolism and in some cases the drug dosage.

Conclusions

The wavelength of 532 nm was the spectrum that best discriminated the absorbance, as it presented better ability to congregate the treatment groups, greater variation between the discriminant and lower coefficient of variation, during the analysis of the colorimetric assay to evaluate the cellular metabolism. The ethanol extract of pequi peel in canine osteosarcoma cells showed that a single dose administration was statistically equal to the reapplication dose every 24 hours.

Methods

The experiment was developed in the Laboratory of Molecular, Cell and Tissue Evaluation, in the Veterinary and Animal Science School of the Universidade Federal de Goiás.

Extract extraction

We used pequi fruit peels collected in the municipality of Nova América, Goiás, Brazil (15.032232º S and 49.942103º W at 730.5 m altitude) processed by Carvalho [11]. The extract was obtained through the fragmentation and drying of exocarp (peel) of Caryocar brasiliense, in a forced-air oven at 40 °C. The samples were milled in razor mills to a size of 20 mm. Then, the samples were weighed and macerated using a mechanical stirrer for 4 hours.

Ethanol was evaporated off on an Ika-Werke rotary evaporator with a water bath at 40 °C. The crude extract was diluted in DMSO (Dimethyl sulfoxide) in a ratio of 1 g/10 ml. The solution was named Ethanol Extract of Pequi Peel (EEPP) and kept in an amber container at -20 ºC. Treatments were prepared by dilution of EEPP in Dulbecco's modified Eagle's medium (DMEM) at concentrations established in the experimental design.

Cell culture

Canine osteosarcoma cells (D-17, BCRJ 0276, Lot 000573, Passage 239, Species Canis familiaris), originating from ATCC (American Type Culture Collection - Manassas, VA, USA), were purchased from the Rio de Janeiro Cell Bank (BCRJ - Rio de Janeiro, Brazil). They were kept in a humidified incubator at 37 ºC and 5% CO2 atmosphere. Cultivation was performed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, plus 1% L-glutamine and 0.1% amphotericin B.

Experimental design

Canine osteosarcoma cells (COC) were seeded in 96 wells plates, at concentrations of 1×10^4/well and exposed to EEPP extract treatments, according to the previously prepared concentrations described in Table 6. The assays were performed in quintuplicate with 3 independent experiments.

Table 6

– Experimental design of MTT colorimetric assay, at concentrations of 1, 10, 100, 1000 µg/mL and control, during the exposure times of 24, 48 and 72 hours to the ethanol extract of pequi peel inoculated into canine osteosarcoma cells of established culture.

Group	**24 hours**						**48 hours**						**72 hours**					
Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Medium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
DMSO		x	x	x	x	x		x	x	x	x	x		x	x	x	x	x
1 µg/mL			x						x						x			
10 µg/mL				x						x						x		
100 µg/mL					x						x						x	
1000 µg/mL						x						x						x

We used 96 well plates with cells at concentrations of 1×10^4 cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% of bovine fetal serum to develop the tetrazolium salt reduction assay (MTT) by the pyruvate dehydrogenase complex present in the mitochondrial matrix. The treatments were randomly distributed, following the homoscedasticity criteria as shown in Fig. 1.

To finish the 24-, 48- and 72-hour treatment at the same time, the wells containing the COC cells were initially treated with the 72-hour group. After 24 hours, the 48-hour group received treatment. And then, after 24 hours, the 24-hour group was treated.

We used 2 independent experiments (repetitions) in quintuplicate to evaluate the effect of treatment application regarding the dosage and exposure time, as described in Table 7. The treatments were randomly distributed, following the homoscedasticity criteria.

Table 7

– Experimental design to evaluate the drug dosage of ethanol extract of pequi peel, at concentrations of 1, 10, 100, 1000 µg/mL and control, during the exposure times of 24, 48 and 72 hours.

Group	**24 hours**					**48 hours**					**72 hours**				
Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Medium	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
1 µg/mL		x					x					x			
10 µg/mL			x					x					x		
100 µg/mL				x					x					x	
1000 µg/mL					x					x					x

The extract application as experimental treatment was performed in two ways. The first group received the application in a single dose per well, following the extract exposure time on the COC cells. The cells remained exposed to the extract for 24, 48 and 72 hours without further extract administration. The second group was subjected to reapplication of the doses prescribed in SID dosage (semel in die), removing the previous extract and adding the same concentration previously established every 24 hours in the exposure groups of 24, 48 and 72 hours.

At the end of the treatment period for both groups, we initiated the tetrazolium reduction assay with 10 µl of tetrazolium (MTT – 3- (4,5-Dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide - Sigma-Aldrich) added to each well. After incubation for three hours, we added 50 µl of 10% sodium dodecyl sulfate (SDS - Vivantis Biochemical) diluted in 0.01N HCL per well. Cells were incubated for 24 hours at room temperature in a light protected incubation chamber. The optical density of each plate was quantified in a spectrophotometer with 532 nm, 540 nm, and 570 nm wavelengths, obtaining three distinct results for the same plate. To better elucidate the MTT assay, a simplified scheme of the main processing steps is shown in Fig. 2.

Statistical analysis

To normalize the data and remove possible outliers, we used a rigorous mathematical model, in which values higher and lower than these intervals were removed from the quintuplicate dataset. Thus, to obtain the threshold for the upper interval, we summed the standard deviation and mean, and for the lower interval, we subtracted the mean with the standard deviation, this model was used independently for each experimental group.

We used randomized blocks as experimental design, using an Analysis of Variance (ANOVA) with Tukey's posthoc test to compare the absorbance means, considering a 5% significance. We performed the analyses using the package easyanova [12] of R software [13].

Abbreviations

MTT: [3-(4,5- dimethyl -2- thiazolyl) -2,5-diphenyl-2H- tetrazolium]

EEPP: Ethanol Extract of Pequi Peel

DMSO: Dimethyl sulfoxide

BCRJ: Rio de Janeiro Cell Bank

ATCC: American Type Culture Collection

DMEM: Dulbecco's modified Eagle's medium

COC: Canine osteosarcoma cells

SID: *semel in die*

HCL: hydrochloric acid

D-17: Canine osteosarcoma cells

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Authors' declare that there are no competing interests.

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Authors' contributions

The authors contributed to the following steps: planning, data interpretation and manuscript writing - LLN, LSC and NPS; experimental design - LLN, VSC, JLF and EA; statistical analysis - EA and JLF; critical review of intellectual content - VSC, JLF, EA and EGA. All authors read and approved the final manuscript.

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References

1. Pantaleão C, Luchs A. Câncer e modelos experimentais de tumores murinos. Revista do Instituto Adolfo Lutz (Impresso). 2010;69 Suppl 4:439-445, from http://periodicos.ses.sp.bvs.br/scielo.php?script=sci_arttext&pid=S0073-98552010000400001&lng=pt&nrm=iss&tlng=pt
2. Bournine L, Bensalem S, Fatmi S, Bedjou F, Mathieu V, Iguer-Ouada M, Kiss R, Duez P. Evaluation of the cytotoxic and cytostatic activities of alkaloid extracts from different parts of Peganum harmala L. (Zygophyllaceae). European Journal of Integrative Medicine. 2017;9:91-96 https://www.sciencedirect.com/science/article/pii/S1876382016303900.
3. Ozi JM, Suffredini IB, Paciencia M, Frana SA, Dib LL. In vitro cytotoxic effects of Brazilian plant extracts on squamous cell carcinoma of the oral cavity. Brazilian oral research. 2011;25 Suppl 6:519-525, from http://www.scielo.br/scielo.php?pid=S1806-83242011000600008&script=sci_arttext&tlng=es

4. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65 Suppl 1-2:55-63, from https://www.sciencedirect.com/science/article/pii/0022175983903034
5. Posimo, J. M., Unnithan, A. S., Gleixner, A. M., Choi, H. J., Jiang, Y., Pulugulla, S. H., & Leak, R. K. Viability assays for cells in culture. JoVE (Journal of Visualized Experiments). 2014; Suppl 83:e50645, from https://www.jove.com/video/50645/viability-assays-for-cells-in-culture
6. Stockert, J. C., Horobin, R. W., Colombo, L. L., & Blazquez-Castro, A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. Acta histochemical. 2018;120 Suppl 3:159-167, from https://www.sciencedirect.com/science/article/pii/S0065128117304749
7. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., & Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer research. 1987;47 Suppl 4:936-942, from http://cancerres.aacrjournals.org/content/47/4/936.short.
8. Rekha, S., & Anila, E. I. In vitro cytotoxicity studies of surface modified CaS nanoparticles on L929 cell lines using MTT assay. Materials Letters. 2019;236:637-639, from https://www.sciencedirect.com/science/article/pii/S0167577X18317683
9. Young, F. M., Phungtamdet, W., & Sanderson, B. J. Modification of MTT assay conditions to examine the cytotoxic effects of amitraz on the human lymphoblastoid cell line, WIL2NS. Toxicology in vitro. 2005;19 Suppl 8:1051-1059, from https://www.sciencedirect.com/science/article/pii/S0887233305000883
10. Vieira GT, de Oliveira TT, Monteiro LP, Kanashiro MM, da Costa MR, Pereira WL. Atividade citotóxica do extrato de Croton urucurana Baill contra linhagens de células leucêmicas humanas U937 e THP1. Ciência e Natura 2017;39 Suppl 3:512-519, from https://www.redalyc.org/pdf/4675/467553545005.pdf
11. Carvalho, L. S. Toxicidade do extrato etanólico da casca de pequi (Caryocar brasiliense Camb.) em camundongos. 86 f. Tese (Doutorado em Ciência Animal) - Universidade Federal de Goiás, Goiânia. 2018;86-86, from https://pesquisa.bvsalud.org/bvs-vet/resource/pt/vtt-210594.
12. Arnhold, E. Package in the R environment for analysis of variance and complementary analyses. Brazilian Journal of Veterinary Research and Animal Science. 2013;50 Suppl 6:488-492, from https://www.revistas.usp.br/bjvras/article/download/55986/pdf_127/.
13. R Core Team R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL R: The R Project for Statistical Computing. 2013;201, from https://repo.bppt.go.id/cran/web/packages/dplR/vignettes/intro-dplR.pdf

Figures

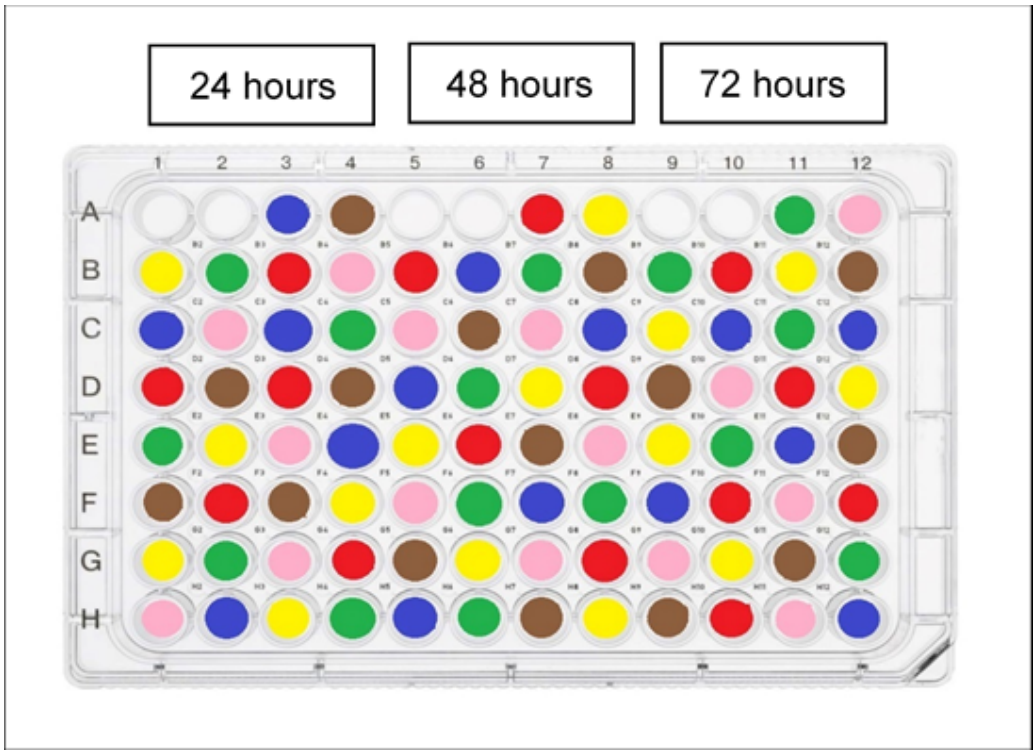

Figure 1

Random distribution of experimental design with canine osteosarcoma cells of stablished culture in 96 wells to perform the MTT colorimetric assay, at concentrations of 1, 10, 1000, 1000 µg/mL and control, during the exposure times of 24, 48 and 72 hours. In yellow, control without SMSO; in red, control with DMSO (0.3 µg/mL); in green, treatment 1 (1 µg/mL); in pink, treatment 2 (10 µg/mL); in blue, treatment 3 (100 µg/mL); in brown, treatment 4 (1000 µg/mL).

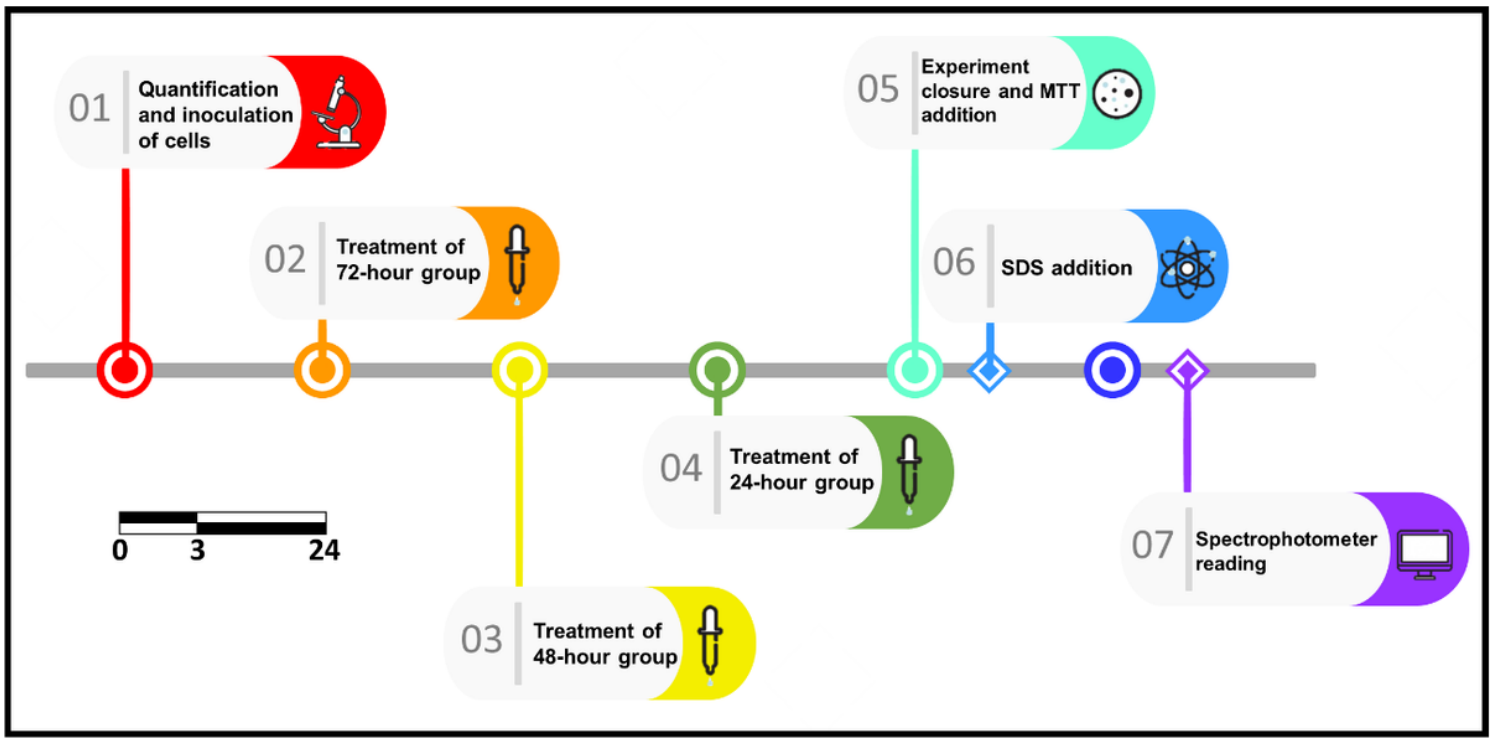

Figure 2

Timeline chart of protocol main steps suggested for the MTT colorimetric assay in 24-hours intervals.