In vitro Cell Viability by CellProfiler® Software as Equivalent to MTT Assay

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Abstract

Objective:

This study evaluated *in vitro* cell viability by the colorimetric MTT stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay compared to image analysis by CellProfiler® software.

Materials and Methods:

Hepatoma (Hepa-1c1c7) and fibroblast (L929) cells were exposed to isolated substances, camptothecin, lycorine, tazettine, albomaculine, 3-epimacronine, trispheridine, galanthine and *Padina gymnospora*, *Sargassum* sp. methanolic extract, and *Habranthus itaobinus* Ravenna ethyl acetate in different concentrations. After MTT assay, cells were stained with Panotic dye kit. Cell images were obtained with an inverted microscope equipped with a digital camera. The images were analyzed by CellProfiler®.

Results:

No cytotoxicity at the highest concentration analyzed for 3-epimacronine, albomaculine, galanthine, trispheridine, *P. gymnospora* extract and *Sargassum* sp. extract where detected. Tazettine offered cytotoxicity only against the Hepa1c1c7 cell line. Lycorine, camptothecin, and *H. itaobinus* extract exhibited cytotoxic effects in both cell lines. The viability methods tested were correlated demonstrated by Bland–Atman test with normal distribution with mean difference between the two methods close to zero, bias value 3.0263. The error was within the limits of the confidence intervals and these values had a narrow difference. The correlation between the two methods was demonstrated by the linear regression plotted as R^2.
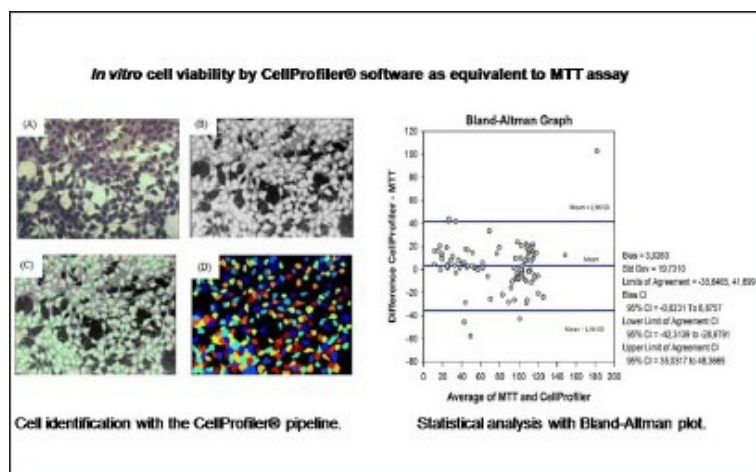
Conclusion:

CellProfiler® image analysis presented similar results to the MTT assay in the identification of viable cells, and image analysis may assist part of biological analysis procedures. The presented methodology is inexpensive and reproducible.

SUMMARY

In vitro cell viability assessment with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay may be replaced by image analysis by CellProfiler®.

The viability methods tested were correlated demonstrated by Bland-Atman test with normal distribution with mean difference between the two methods close to zero, bias value 3.0263. The correlation between the two methods was demonstrated by the linear regression plotted as R2.

In vitro cell viability by CellProfiler® software as equivalent to MTT assay

Cell identification with the CellProfiler® pipeline.

Statistical analysis with Bland-Altman plot.

Abbreviations: HPLC: High pressure liquid chromatography MTT: (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)

Keywords: CellProfiler®, cell viability, Hepa-1c1c7, L929, image analysis, MTT

INTRODUCTION

The common techniques applied for determination of *in vitro* cell size, morphology and growth normally involve human manual work, which is imprecise and frequently subject to variability caused by the analyst himself or herself.[1] In addition, considering the evaluation necessity of a large amount of material and data, automatic image analysis is very desirable.[2] The use of accessible software for

determination of cells morphology and viability allows accurate measurement where errors become standardized as it is performed automatically.[3] Automated image analysis has several advantages compared to manual analysis,[4,5] including speed, objectivity, quantitative and reproducibility. [4,5,6,7]

CellProfiler® software is freely available to download at www.cellprofiler.org. It is an automated image analysis for identification of cells and analyses converting the identified cells in digital images. After counting and recording the cells, it creates a wide spectrum of data such as cellular and complex morphologic analysis as for staining, size and morphology.[8] This automated method generates highly reproducible assays and performs cell selection and differentiation at a lower cost as it uses in turn, less reagents than traditional manual methods.[4] In addition, CellProfiler® optimizes the analysis time spent[4,5,9] and it possesses functionality and throughput, being also possible to integrate with other open-code software.[10] It also performs biological phenotypes measurements automatically and quantitatively from a number of images.[11] It is easy to be used by researchers who do not have great computer skills, good for implementation of methodology based on image analysis.[12] The program uses an algorithm to create a sequence of configurations to process the image according to the researcher's criteria.[8] It has been reported as a method considered easy, low cost, and efficient to detect necrosis and apoptosis.[13] CellProfiler® proved to be an effective facilitator of biological analyses, including the analysis of parameters in mouse retina cells[9] chromosome measurements,[14] a pilot study of cellular analysis in bird hemograms[15] and the identification of *Chlamydia* species, in a much shorter time.[16] Bray and collaborators[5] developed and used a pipeline for the identification of yeast colonies in agar plates, confirming the possibility to create adjustable pipelines in several biological experiments.

Evaluation and triage methods for artificial or natural substances having cytotoxic activity are relevant for antitumor treatment.[17,18] Several plants are constantly analyzed to verify their phytochemical composition and potential in pharmaceutical science[19]. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) colorimetric assay is a very common tool for cytotoxicity evaluation as a mean to find new drugs to eliminate cancer cells. This was the methodology used by Samarghandian *et al*.,[20] against breast cancer cells (MCF-7); by Sibi and Rabina[21] against RAW 264.7 cells; by Siddiqui *et al*.,[22] for breast cancer cell lines; CT26 and by Mathew *et al*.,[23] against hepatocellular carcinoma. MTT is also used to verify protective effect as it evaluates cell viability in turns.[24]

Considering that 100 alkaloids structures have been isolated and elucidated from *Amaryllidaceae* family plant, with antiproliferative activity over eukaryotic cells by interfering with protein synthesis, [25] this work proposes to select isolated alkaloids from this family and to evaluate cytotoxicity comparing the traditional colorimetric MTT method with image data generated by CellProfiler®. Isolated alkaloids from the *Amaryllidaceae* family as licorine, 3- epimacronine, tazettine, trispheridine, albomauline, and galanthine. In addition, camptothecina, a well-known cytotoxic substance and tree extract, algae *Padina gymnospora* methanolic extract, *Sargassum* sp. algae methanolic extract, and a plant *Habranthus itaobinus* Ravenna ethyl acetate extract were also included for cytotoxicity evaluation.

MATERIALS AND METHODS

Cell Line and Reagents

Hepatoma cancer Hepa-1c1c7 cells (ATCC® CRL-2026) and fibroblast L929 cells (ATCC®CCL1TM) were kept on Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, USA) with 3.7 g NaHCO$_3$ and supplemented with 10% fetal bovine serum (FBS, Cripion, Brazil), 60 µgm^{L-1} penicillin,

and 100 μgm^{L-1} streptomycin (Sigma-Aldrich, USA). The cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2). Camptothecin and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma Aldrich®. Panotic kit staining was purchased from New-Prov Brazil. The isolated substances and extracts tested in this study were provided by Paulo Cezar Vieira from the Laboratory of Natural Products (Federal University of Espírito Santo and University Vila Velha, Brazil). Reagents and chemicals used were of analytical grade.

Cellular Treatment for Image Analysis and MTT Assay

Hepa-1c1c7 and fibroblast L929 cells were seeded at a density of 7×104 cells mL^{-1} in 96-well plates and cultured for 24 hours with increasing concentrations of camptothecin (0.045-100 μM), *Padina gymnospora* algae methanolic extract (0.300-312.5 μgmL^{-1}), *Sargassum* sp. Algae methanolic extract (0.300-312.5 μgmL^{-1}), lycorine (0.1-83 μM), tazettine (0.03-30 μM), *Habranthus itaobinus* ethyl acetate extract (0.08-312.5 μgmL^{-1}), albomaculine (0.75-46 μM), 3-epimacronine (3.03-19 μM), trispheridine (4.50-28 μM), and galanthine (0.88–56 μM). After 24-hour incubation, the medium was removed and the cells were stained either Panotic staining for the CellProfiler® analysis or MTT for the colorimetric viability assay.[26]

Image Analysis Preparation

The medium was removed and cell fixation and coloration were performed with Panotic kit solution I, II and III following the manufacturer instructions. As the cells were on plate, and not on slide, a volume of 50 μL for each solution was applied.

Thereafter, 10 photos were taken for each sample concentration with an inverted microscope (LGD3 model, Eikonal, Brazil). Cell analysis was performed using the CellProfiler® software for cell parameters.

Image Analysis and Mobile Identification

For each concentration evaluated, the same standard analysis was used for the pipeline (step by step) [Table 1]. The analysis only changed in identifying the primary-object item, minimum area, and maximum area, depending on the need of each concentration. The images were identified, analyzed, and quantified by CellProfiler® [Figure 1]. The recognized cells are illustrated by Figure 2, L929 cells (A), Hepa-1c1c7 cells (B).

Table 1

Configuration of sequence pipeline in CellProfiler® software

1 Load Images
2 Color to Gray
3 Image Math
4 Identify Primary Objects - Cell
a) Typical Diameter of Objects Min-Max: 20-200 pixels
b) Threshold Strategy: Adaptive
c) Thresholding Method: Robust Background
5 Measure Object Intensity
6 Measure Object Size/Shape

From these data, it is possible to identify the core, intensity, area, and shape of the charged objects.
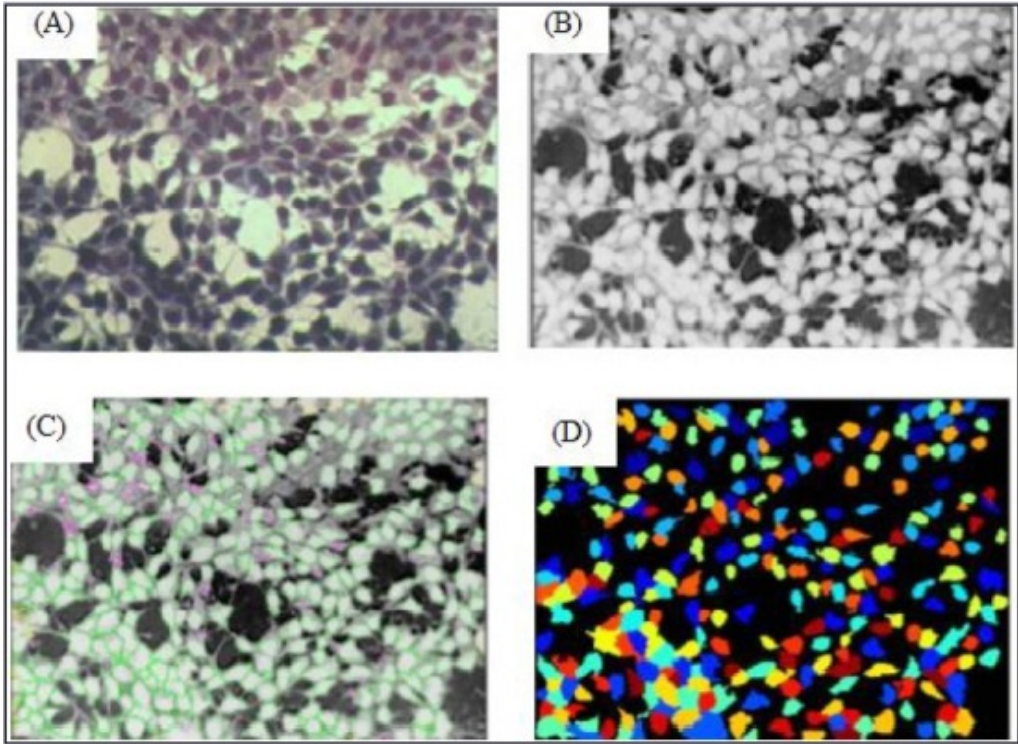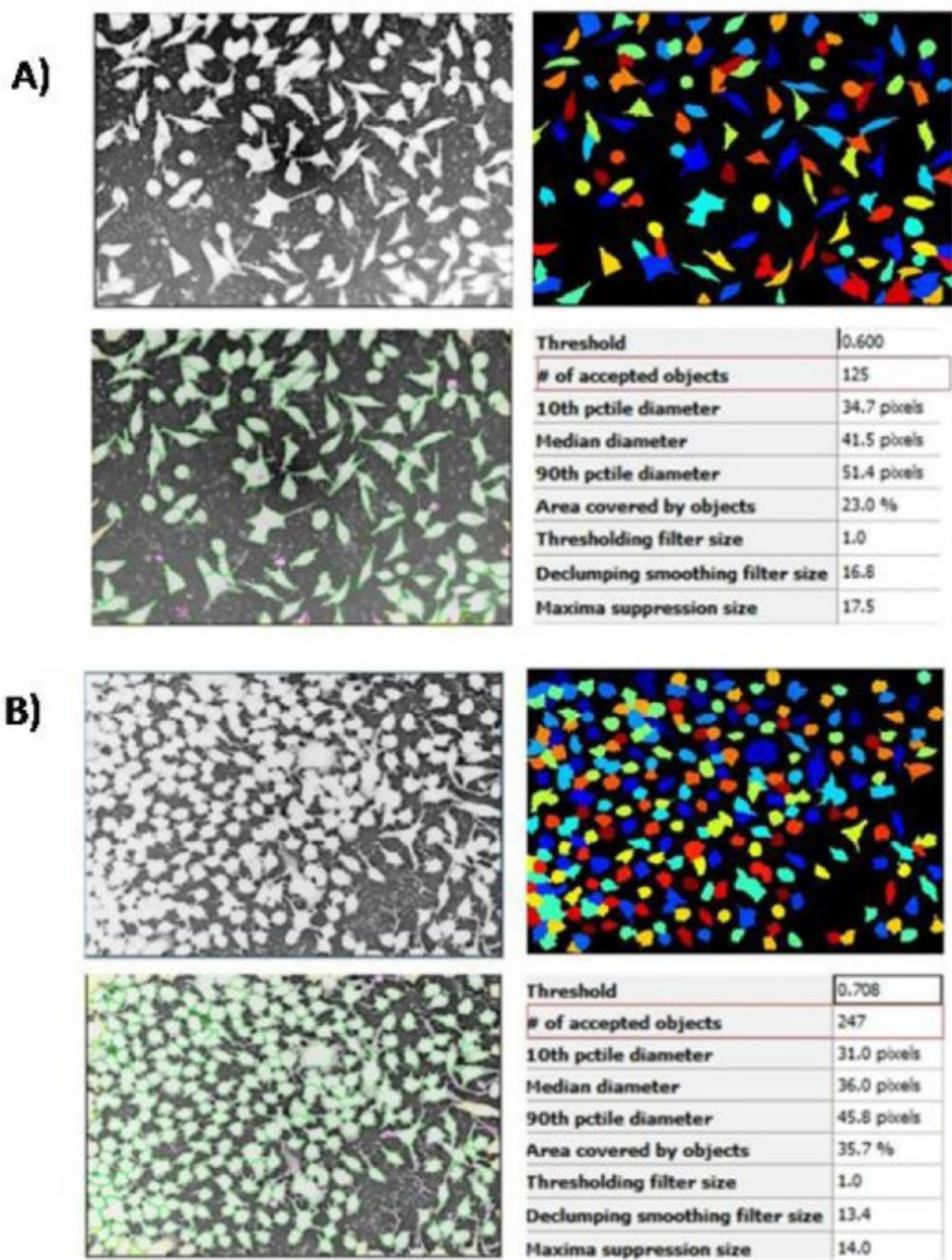

Figure 1

Cell identification with the CellProfiler® pipeline Figure 1: (A) The original image. (B) Image converted to grayscale. (C) In green, identified cells according to the established parameters (size and shape); in pink cells deleted (because are not within the parameters). (D) Cells identified from picture c, each colored point represents one identified cell

Threshold	0.600
# of accepted objects	125
10th pctile diameter	34.7 pixels
Median diameter	41.5 pixels
90th pctile diameter	51.4 pixels
Area covered by objects	23.0 %
Thresholding filter size	1.0
Declumping smoothing filter size	16.8
Maxima suppression size	17.5

Threshold	0.708
# of accepted objects	247
10th pctile diameter	31.0 pixels
Median diameter	36.0 pixels
90th pctile diameter	45.8 pixels
Area covered by objects	35.7 %
Thresholding filter size	1.0
Declumping smoothing filter size	13.4
Maxima suppression size	14.0

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Figure 2

Identification and quantification of objects (cells) by CellProfiler® software. L929 cells (A), Hepa-1c1c7 cells (B)

Colorimetric cell viability assay

In vitro cytotoxic activity was evaluated by the colorimetric MTT assay.[26] Briefly, the MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is cleaved into a blue colored product (formazan) by the mitochondrial enzyme succinate-dehydrogenase. Dissolution of the formazan crystal is performed by addition of 100μL dimethyl sulfoxide. The absorbance was determined at 595 nm using a microplate reader (Spectra Max 190, Molecular Devices, USA).

The Hepa-1c1c7 and L929 cells were plated in 96-well, flat-bottom plates at a concentration of 0.7×10^5 cells mL^{-1}, overnight incubated at 37°C at 5% CO_2 in-air atmosphere. Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was used for cell cultivation. The cells were exposed to different concentrations of the substances and extracts.

To calculate cell viability, the absorbance resulted from the DMSO is dimethyl sulfoxide (0.5%) control was used as 100% cell survived been comparing to the absorbance of samples tested as "x" and expressed as mean and standard deviation.

The cytotoxic substances have their half maximal inhibitory concentration IC_{50} estimated with Table Curve 2D$^®$ software.

Statistical Analysis

To evaluate the correlation between the two methods used, a Bland–Altman analysis was applied with Sigma Plot Systat Software Inc. To be considered as acceptable results for Bland–Altman analysis, the data must have a normal distribution and good correlation between the two methods.[27] An average close to zero indicates that methods were good correlated between them.

RESULTS AND DISCUSSION

Compounds that presented selectivity for cancer cells are very important in chemotherapy novel drug development. Camptothecin was identified as an alkaloid substance that efficiently impairs cancer cells growth.[28] It was isolated from *Camptotheca acuminado* a plant with China origin.[29,30] Li *et al*.[28] showed an IC_{50} of 0.56 μM for camptothecin in a human liver cancer line and Piao *et al*.[31] found an IC_{50} of 0.70 μM for the same lineage. In addition, Fronza *et al*.[32] showed an IC_{50} of 0.4 μM in the pancreatic cancer cell lines. The present study showed an IC_{50} of 0.47 μM for camptothecin in Hepa-1c1c7 and 0.7 μM in L929 cells similar to the data previously reported. Camptothecin was commercially acquired and was selected as standard to compare the other substances to be tested.

Among the isolated substances and considering the traditional MTT method for toxicity, lycorine presented IC_{50} for cancer cells Hepa1c1c7 of 2.88 μM (dp 0, 03) and for the normal fibroblasts L929 cells of 0.62 μM (dp 0.02). Tazettine presented cytotoxicity only against Hepa-1c1c7 (IC_{50} = 10.40 μM). Luo *et al*.[33] evaluated alkaloids cytotoxicity as 3-epimacronine, tazettine, trispheridine, and lycorine. They reported cytotoxic only for lycorine against leucocytes cancer cells HL60 with IC_{50} of 2,07 μM (dp 0,3). The results presented agree with the cytotoxicity for lycorine against Hepa1c1c7 cancer cells. *Habrantus* species from the *Amaryllidaceae* family are known as alkaloids produces that differentiate between them in the chemical structure. They have been reported as antitumor and cytotoxic.[34] In the present study, *H. itaobinus* presented IC_{50} of 0.78 μgmL^{-1} (dp 0.09) for Hepa 1c1c7 and 0.14 μgmL^{-1} (dp0.003) for L929 cells. The IC_{50} data here demonstrated that the cells evaluated are more sensible to the *H. itaobinus* than the cancer cells HL60 that presented IC_{50} of 11,5 μgmL^{-1} against *H. brachyandrus* reported by Jitsuno.[34] In addition, 3-epimacronine, albomaculine, galanthine, and trispheridine were tested against Hepa1c1c7 and normal cells L929 fibroblasts in the concentration of 19, 46, 56, 28 μM respectively. They did not present cytotoxicity in these concentrations. Bessa[25] also reported no cytotoxicity for albomaculine against mammal's cells.

Considering the algae extracts *P. gymnospora* did not present cytotoxicity in the concentration tested of 312.15 μgmL^{-1} with 99.40% (dp11.39) viability for L929. The positive cell proliferation activity could also be demonstrated for methanolic extract of these algae.[35] In addition, the methanolic extract of *P. pavonia*, brown algae, demonstrated low cytotoxicity for normal lung human cells MRC-5 of IC_{50} > 200 μgmL^{-1}.[36] *Sargassum* sp. evaluated in a concentration of 312.15 μgmL^{-1} also had no

cytotoxicity. However, *Sargassum angustifolium* presented cytotoxicity effect against T47D and HT 29 cell lines with a IC_{50} of 166.42 ± 26.7 and 190.24 ± 52.8 µgmL^{-1}.[28] However, alginate isolated from *Sargassum fulvellum* demonstrated antitumor effect against murine tumor Sarcoma 180 (no cystic and solid form), and Erlich carcinoma.[36]

Cell viability was assessed by image analysis with CellProfiler® software and compared with data from cell viability determined by colorimetric MTT assay. The results are presented as percentage of cell survival [Table 2]. The data had normal distribution confirmed by histogram, and the mean difference between the two methods was close to zero, as shown by the bias value 3.0263. Moreover, 95% of the values were between the established concordances limits -35.6465 and 41.6991. The error was within the limits of the confidence intervals and these values had a narrow difference. The correlation between the two methods was demonstrated by the linear regression plotted as R^2.
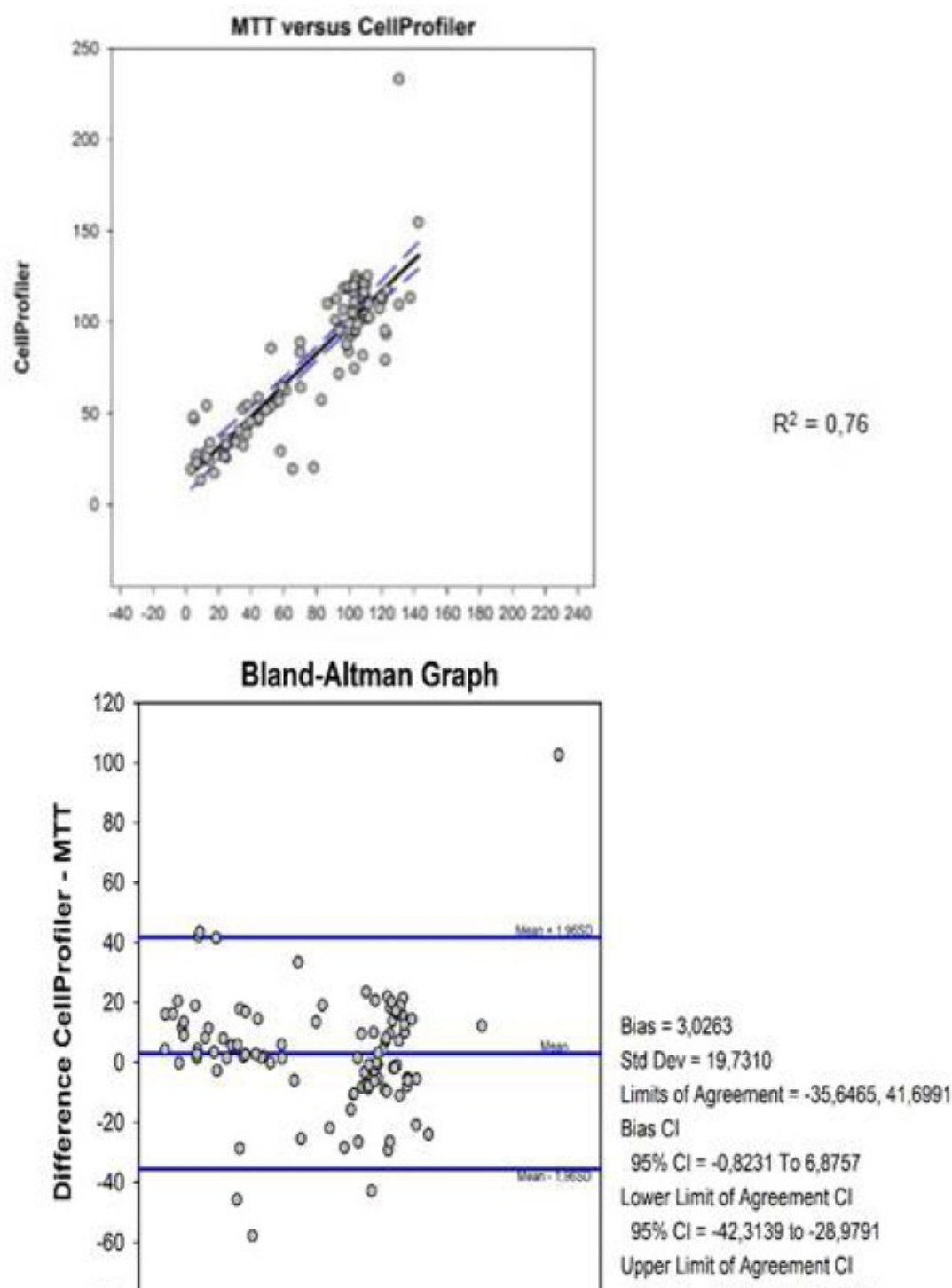
Table 2

Cellular viability of tested substances at their maximum concentration tested

Sample	Concentration	Hepa-1c1c7		L929	
		MTT (CV)	CP	MTT(CV)	CP
3-epimacronine	19 µM	102 (2.13)	97 (2.05)	111.14 (1.80)	101.95 (9.32)
Albomaculine	46 µM	103.28 (2.60)	74.75 (4.33)	103.56 (6.52)	108.63 (2.44)
Galanthine	56 µM	103.85 (3.07)	125.23 (4.78)	112.29 (9.22)	102.56 (3.66)
Trispheridine	28 µM	98.30 (1.32)	87.50 (4.23)	105.22 (5.28)	98.92 (2.36)
Tazettine	30 µM	34.87 (1.90)	52.59 (4.99)	142.54 (9.50)	154.64 (1.39)
Lycorine	83 µM	6.71 (1.12)	27.05 (1.77)	6.71 (1.12)	22.87 (1.03)
Camptothecin	100 µM	65.42 (0.59)	19.54 (1.22)	9.12 (8.14)	13.45 (7.26)
Padinagymnospora	312.5 µgmL^{-1}	100.07 (9.17)	99.18 (1.20)	99.40 (11.39)	119.47 (7.33)
Sargassum sp.	312.5 µgmL^{-1}	108.35 (2.39)	81.73 (1.36)	102.05 (6.60)	105.15 (3.62)
Habranthusitaobinus Ravenna	312.5µgmL^{-1}	17.52 (0.61)	17.23 (1.99)	12.75 (030)	26.15 (1.67)

Results are expressed as the mean (%) ± standard deviation of three independent experiments. MTT assay, CellProfiler® analysis.

Comparing the methodologies, MTT and image analysis, the study presented significant and acceptable results in the Bland–Altman test. The data presented a normal distribution [Figure 3]. The histogram data confirmed the normality, and the graph showed no distortion or very long tails.[37] Altman and Bland[34] recommended that 95% of the figure points be within the difference of means in the standard deviation, and the closer to zero the difference of means is, the greater the agreement is. In this study, the difference between the averages was 3.0263, being very close to zero as recommended by the authors.

MTT versus CellProfiler

$R^2 = 0{,}76$

Bland-Altman Graph

Bias = 3,0263
Std Dev = 19,7310
Limits of Agreement = -35,6465, 41,6991
Bias CI
  95% CI = -0,8231 To 6,8757
Lower Limit of Agreement CI
  95% CI = -42,3139 to -28,9791
Upper Limit of Agreement CI

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Figure 3

Statistical analysis with Bland-Altman plot Figure 3: This figure shows the correlation of cell viability as calculated by CellProfiler® and MTT assay

According to Giovarina,[37] the confidence interval (CI) checks how precise the estimates of the data are and allows one to estimate a possible sampling error. The larger the sample size is, the narrower the confidence interval and the greater the reliability will be for the data to correspond to the true values. The data presented in this study showed narrow CI values, between -35.6465 and 41.6991, suggesting that the values were approximately acceptable of the true value. Therefore, the data showed an acceptable standard statistical correspondence between the feasibility of the MTT method and analysis with CellProfiler®.

The correlation coefficient is an association measurement, not being appropriate to evaluate the concordance between methods.[38,39] The Bland–Altman method is the most adequate to assess this concordance [Figure 3].[40] According to the assessment of this study's data, the image analysis is promising in the analysis of cellular viability, presenting a statistical significance P< 0,5 among the obtained values.

CONCLUSIONS

Image analysis with CellProfiler® software proved to be a fast method to optimize cell analysis. This technique allowed the verification of cellular morphology and presented with little variation, good correlation with the gold standard test MTT. In addition, the image analyses were reproducible being an efficient method. The present study used only routine staining, a low-cost method to evaluate viability and cellular parameters. Without the need of a fluorescent staining or other techniques that elevate the final cost, image analysis is a convenient alternative to visual analysis. This study provided a quick, inexpensive, and efficient analysis. However, the scarcity of data on this subject denotes the importance of more studies to enhance this technique.

Ethical Approval

The authors did not use any human or animal participants in this study or preliminary studies.

Conflicts of interest

There are no conflicts of interest

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