Original Research Paper

Hybrid design as a strategy for development of trypanocidal drugs

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ABSTRACT

Aims: We performed an initial in vitro study with a single molecule to evaluate the possibility to develop a larger series of hybrid compounds active in Chaga's Disease. Hybridization is an important approach to confer to a single molecule the biological activity of two distinct molecules. We proposed thiosemicarbazone (TS9) hybridization with β-citronellol through carbamate linkage.

Methodology: The citotoxicity of the hybrid compound was evaluated against human THP-1 cells and all forms of *T. cruzi.* IC_{50} value was determined against amastigotes and selectivity index (SI) was estimated based on toxicity against THP-1 cells. Lipinski analysis was performed in order to estimate the hybrid drug-like properties.

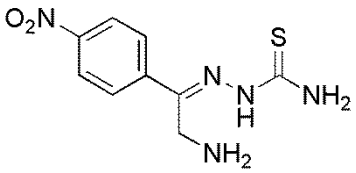
Results: The hybrid presented substantially less cytotoxicity against THP-1 cells than TS9 and biological similarities to both matrix moieties. The hybrid SI (3.9) was better than for TS9 (0.6) and similar to that found for benznidazole (4.7), but with higher drug-like score performed by Lipinski analysis.

Conclusion: In face of its relevant trypanocidal action against *T. cruzi* amastigotes, it's an important concept proof to pursue in develop of hybrid or prodrug derivatives of TS9 and antiprotozoal terpenes.

Keywords: thiosemicarbazone; hybrid compound; Trypanosoma cruzi; terpenes; mixed carbamate.

1. INTRODUCTION

The parasitocidal action of thiosemicarbazones has been explored in several works, emphasizing its importance in inhibiting cysteine proteases, as cruzipain from *Trypanosoma cruzi* [1], rhodesain from *T. brucei rhodesiense* [2], falcipain-2 *from Plasmodium falciparum* [3], and CPB from *Leishmania Mexicana* [4]. From the work of Du *et al*. (2002) [1], several molecular changes have been proposed trying to optimize its action on cruzipain. In the year of 2013, our group published a work providing several acetophenone thiosemicarbazone derivatives. One of them, the compound 2-amino-1-(4-nitrophenyl) acetophenone thiosemicarbazone (TS9), showed a high activity against epimastigote forms of *T.cruzi* [5]. This compound presents a primary amine group at position 2 (**figure 1**), which was able to suffer further derivatizations leading to hemissuccinic acid derivatives, that retained its trypanocidal activities. At this point, it is noticed that from the exploration of position 2 of acetophenone thiosemicarbazones, emerges the possibility to develop hybrid compounds possessing better pharmacokinetics and toxicological profiles. In the present work, we proposed the obtainment of the 2-*N*-carbamoyl terpene derivative of 2-amino-1-(4-nitrophenyl) acetophenone thiosemicarbazone (TS9) aiming lowering cytotoxicity of the TS9 compound and potentiating its trypanocidal action against amastigote form of *T. cruzi.* (**figure 2**).



2-amino-1-(4-nitrophenyl)
acetophenone thiosemicarbazone (TS9)

FIG. 1. Molecular structure of 2-amino-1-(4-nitrophenyl) acetophenone thiosemicarbazone (TS9)

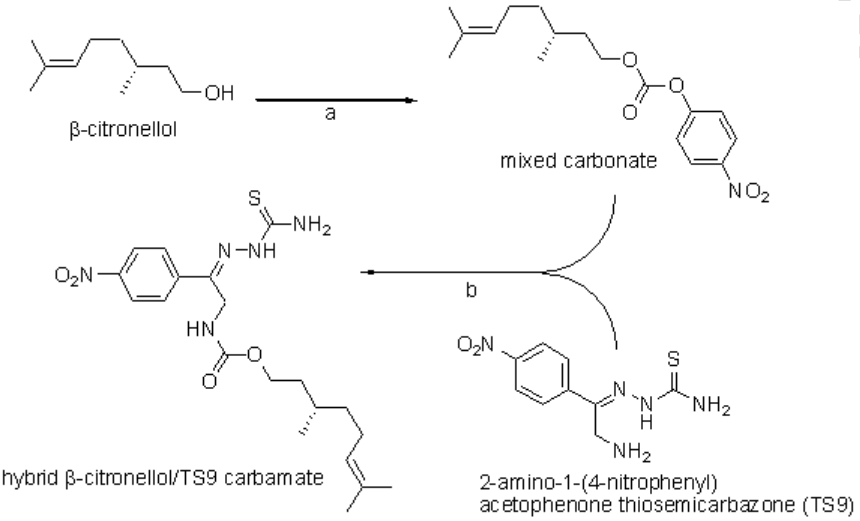


FIG. 2. Synthetic rout for obtainment of hybrid carbamate terpenic derivatives. a. *p*-nitrophenyl chloroformate; DMAP; 2,6-lutidine; THF; 24h; rt. b. DIPEA; CH2Cl2; 6h; rt.

The terpene (β-citronellol) was selected due to its previously described antiprotozoal actions under *in vitro* conditions without, however, the knowledge about the biochemical pathway of its antiprotozoal activity [6-9]. Several trypanocidal terpenes were not able to inhibit cruzain (G. Trossini and R.F. Menegon, unpublished data), a recombinant form of *T. cruzi* cruzipain, revealing that their trypanocidal activities involve a different action than those described for thiosemicarbazones. This finding leads us to propose a hybrid of β-citronellol and TS9 possibly presenting a dual trypanocidal activity, minimizing the possibilities of parasite resistance and improving pharmacokinetics parameters, as oral bioavailability, by balancing the lipophilicity and hydrophilicity of both terpenic and TS9 moieties. The strategy of hybrid formation has been used in molecular modeling to improve both biological activity of antimicrobial compounds [10,11] and drug resistance in antimalarial chemotherapy [12].

The linkage of both active biological moieties into the hybrids involves a carbamate group. Carbamates present relative enzymatic and chemical stability at physiological conditions, but low stability under acidic medium [13, 14]. This property brings up the possibility of the hybrid to keep unchanged in plasma, but once in the inner of macrophages, the acidic medium promoted by the lysosomal activity could prompt the hydrolysis of the carbamate, delivering both free terpene and thiosemicarbazone moieties. Once macrophages play an essential role in the life cycle of *T. cruzi*, this site for hybrids cleavage could be the aim for prodrug development in further works [14, 15].

2. MATERIAL AND METHODS

2.1 Synthesis of the Hybrid Carbamate

Compound TS9 (2-amino-1-(4-nitrophenyl) acetophenone thiosemicarbazone) was obtained according to Blau *et al*. (2013) [5], and its chemical structure was confirmed by comparing melting point and ^{1}H NMR to the previously described [5, 16].

Carbamate synthesis of hybrid β-citronellol/TS9 was carried out by two synthetic steps: 1. The formation of a mixed carbonate of β-citronellol from *p*-nitrophenyl chloroformate [17]; and 2. Nucleophilic substitution of *p*-nitrophenyl moiety by thiosemicarbazone (TS9).

The coupling reaction of the mixed carbonate to compound TS9 was carried out employing a constant alkaline medium, kept at pH 9.0 to 10.0 with additions of drops of anhydrous N,N-diisopropylethylamine (DIPEA) along the reaction. The basic medium was necessary due to zwitterion conformation assumed for compound TS9, whose aliphatic amine group is able to form a dipolar ion with the acidic imine hydrogen that, in your turn, can inhibit the carbamate formation. Hybrid carbamate was obtained in moderate yield (53%) after purification by silica gel liquid chromatography. Detailed information concerning synthesis and structural characterization of the hybrid carbamate may be found in the appendix section.

β-citronellol was purchased from sigma-aldrich, and are not enantiomeric pure. All other reagents, α-brome-*p*-nitroacetophenone (acros), thiosemicarbazide (acros), hexamethylenetetramine (synth), N,N-dimethyl-4-aminopyridine (Flucka), N,N-diisopropylethilamine (sigma-aldrich), 2,6-lutidine (sigma-aldrich), Prestoblue™ (invitrogen), are ACS grade or more than 97% of purity and were used with no further treatment. The solvents employed for synthesis was purchased from Synth and dried according to Vogel (1989) [18].

2.2 Biological Assays

2.2.1 *In vitro* cytotoxicity on THP-1 cell

The *in vitro* cytotoxicity on leukemic monocyte THP-1 cell line human (ATCC® TIB-202) was performed with PrestoBlue™ (Invitrogen) cell viability reagent, following the manufacturing instructions. 5.0 x 10^5 cells/well were incubated for 24 hours at 37°C in plates of 96 wells with the selected compounds at concentration ranging from 100 μg to 1.562 μg to a final volume of 200 μL. After 24 hours, 10 μL of PrestoBlue™ reagent was added over 90 μL of cell supernatant. After 2 hours at 37°C, the fluorescence intensity was measured in base of relative fluorescence units (RFU) in a Spectramax® M3 device at wavelengths 560 nm (excitation) and 590 nm (emission). The assay was performed in duplicate.

2.2.2 *In vitro* evaluation of the trypanocidal activity against trypomastigote and epimastigote forms.

The *in vitro* evaluation of the trypanocidal activity against trypomastigote and epimastigote forms was performed with PrestoBlue™ (Invitrogen) cell viability reagent. 1.0 x 10^7 trypomastigotes/well were incubated for 1 and 2 hours at 37°C in plates of 96 wells with the compounds at concentration ranging from 100 μg to 0.78125 μg to a final volume of 500 μL. 1.0 x 10^7 epimastigotes/well were incubated for 1, 3, 6, 24 and 48 hours at 37°C in plates of 96 wells with the compounds at concentration ranging from 100 μg to 0.78125 μg to a final volume of 500 μL. After the incubation time, 10 μL of PrestoBlue™ reagent was added over 90 μL of supernatant. After 2 hours at 37°C, the fluorescence intensity was measured in base of relative fluorescence units (RFU) in a Spectramax® M3 device at wavelengths 560 nm (excitation) and 590 nm (emission). The assay was performed in duplicate.

2.2.2 *In vitro* evaluation of the trypanocidal activity against amastigote forms.

1.0 x 10^5 leukemic monocyte THP-1 cell line human were infected by trypomastigotes forms in a multiplicity of infection (MOI) of 30 parasites: 1 cell. 24 hours after infection cells, as well as parasites, were incubated for 24 hours at 37°C in plates of 24 wells, with circular coverslip 13 mm (Glasscyto), with the determined compounds at concentration ranging from 100 μg to 6.25 μg to a final volume of 500 μL. After 24 hours, coverslips were fixed with Bouin's solution (Sigma Life Science) and stained with Giemsa's azur eosin methylene blue solution (Merck). Coverslips were mounted with Entellan® New (Merck) on microscope slides 26x76 mm (Perfecta). To determine the trypanocidal activity against amastigote forms, 20 fields/slide were counted. In each count were determined: number of total cells, number of infected cells and number of intracellular parasites (amastigotes). The assay was performed in duplicate.

2.2 Statistics

Statistical analysis were performed by One-Way ANOVA test, and complemented by LSD posthoc test (α = .05), employing Statistica software version 13.3 (TIBCO software Inc). IC_{50} values against amastigote forms were estimated by linear regression curve from logarithm of concentration (μg/500μL), and further converted to μM concentration.

3. RESULTS AND DISCUSSION

From **table 1**, it's observed that compound TS9 exhibits the highest toxicity against THP-1 cells over all tested compounds, making its use possible only at concentrations below 1.56 μg/200 μL (7.8 μg/mL). Albeit the high toxicity of TS9, its hybrid carbamate derivative diminishes TS9 toxicity by more than 20 times. Comparing its maximum non-toxic concentration (574.00 (+/- 0.32) μM) with those observed for TS9 (26.92 (+/- 0.10) μM) and for β-citronellol (1599.80 (+/- 0.26) μM), cytotoxicity of the hybrid against THP-1 cells reaches an intermediate position between them.

Table 1. Maximum tested concentration with no evidence of toxicity against THP-1 cells

Compound	Maximum non-toxic concentration	Non-toxic µM concentration (+/- SD)
TS9	1.562 µg/200µL	26.92 (+/- 0.10) µM
β-citronellol	50 µg/200µL	1599.80 (+/- 0.26) µM
Hybrid carbamate	50 µg/200µL	574.00 (+/- 0.32) µM
Benznidazole	50 µg/200µL	960.61 (+/- 0.31) µM

It was not possible to determine an IC_{50} value against THP-1 cell from the tested concentration range, once all compounds but TS9 demonstrated no evidence of toxicity except for the highest concentration on 50 µg of compound diluted in 200 µL of cell suspension (**figure 3**).

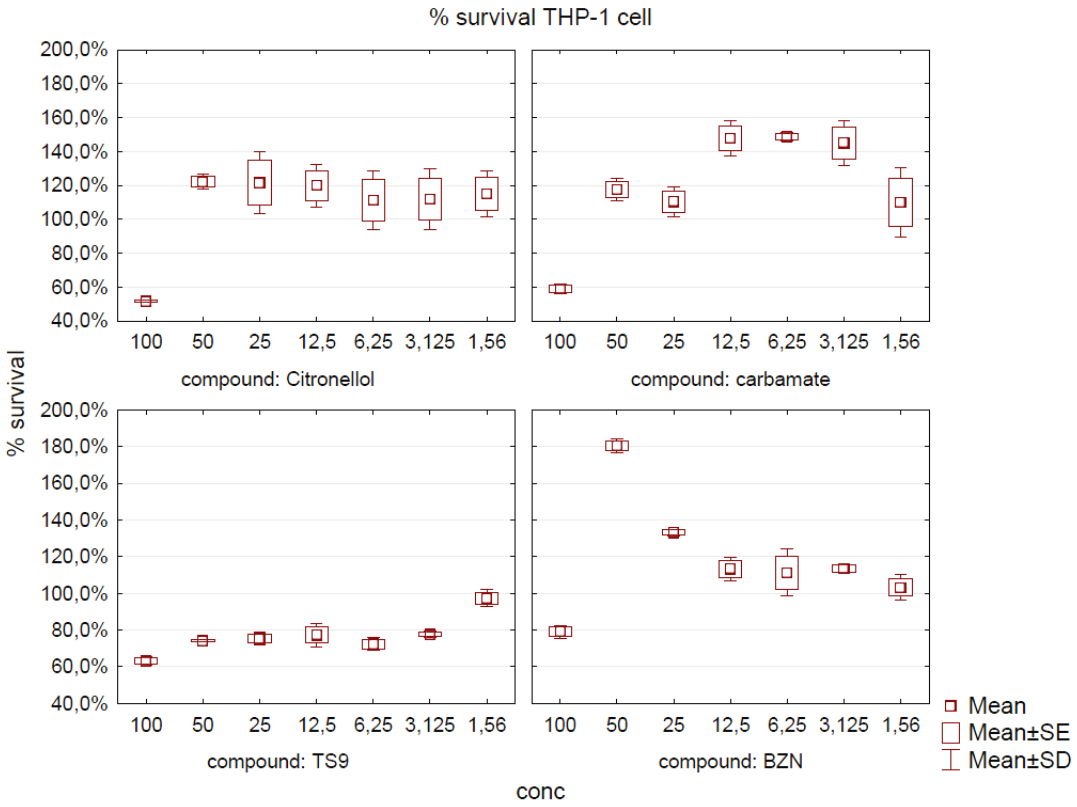


FIG. 3. Anti-proliferative effect of β-citronellol, hybrid carbamate, TS9 and benznidazole agaist THP-1 cell lineage versus concentration (µg/200µL).

It's notorious the β-citronellol moiety influence on citotoxicity profile of the hybrid carbamate. From **figure 4(a)** we can observe that beside there is no statistic difference

between TS9 and hybrid carbamate (*P* = .65), the average of percentage of survival cell of the carbamate occupies an intermediate position between TS9 and β-citronellol values at the maximum tested concentration (100 µg), where β-citronellol exhibits the maximum toxic effect. However, when the concentration is diminished to 50 µg (**figure 4(b**)), there is a complete inversion of this order, and while TS9 keeps a similar inhibition of cell growth, both β-citronellol and its hybrid carbamate citotoxicity is strongly diminished, presenting no toxic effect against THP-1 cell lineage. No evidence of anti-proliferative effect of all tested compounds is observed only at the minimum concentration of 1.56 µg (**figure 4(c**)). The high toxicity of TS9 can be easily observed when we analyze the overall toxicity grouping all concentrations in the same graph (**figure 4(d**)). TS9 present an average of 76.81% of survival cells differing from the average of 107.60% for β-citronellol (*P* = .003), 116.71% for hybrid carbamate (*P* < .001) and 119.12% for benzonidazole (*P* < .001). No statistic difference were observer between the last three groups (*P* > .2).

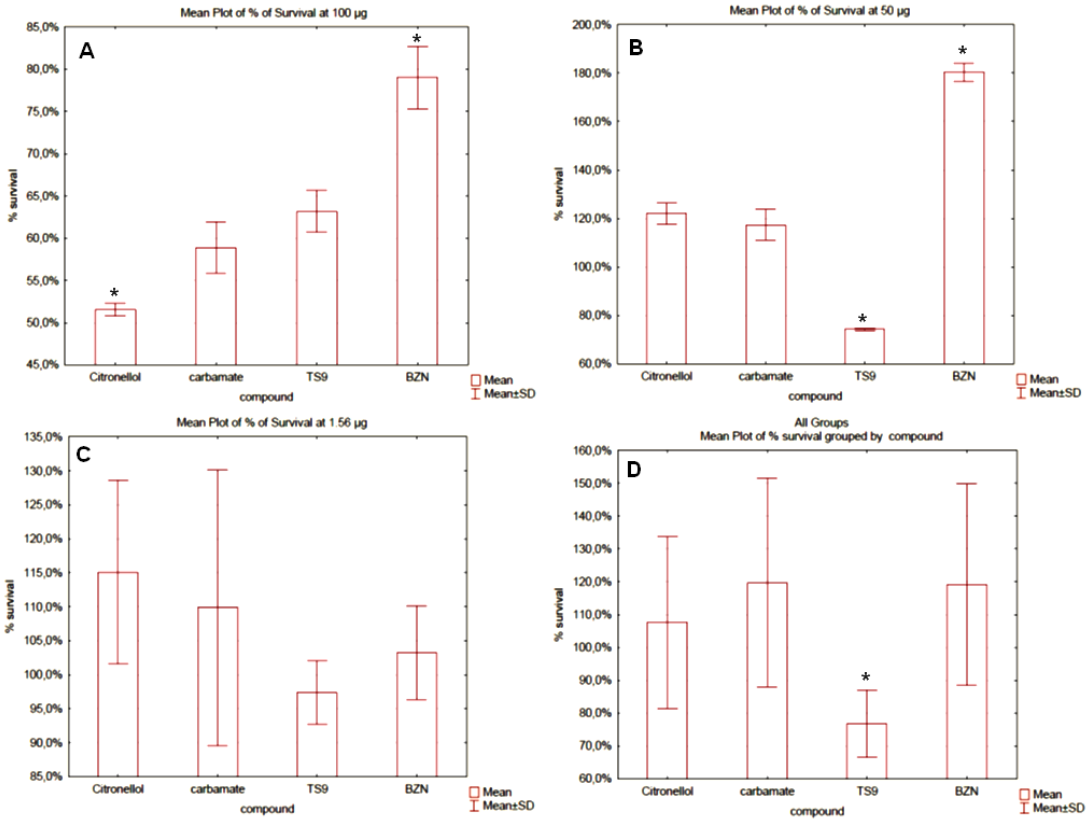


FIG. 4. Citotoxicity against human macrophage THP-1 cell lineage. a) 100 µg/200µL; b) 50 µg/200µL; c) 1.56 µg/200µL and d) overall average of all tested concentrations.

To investigate the trypanocidal action of the hybrid carbamate, anti-proliferative action againt epimatigotes, trypomastigotes and amastigotes forms of *T. cruzi* Y-strain were tested. Epimastigote forms of *T. cruzi* are restricted to the triatomine vector, and are not found in humans. Despite the convenience of this test, concerning its safety on manipulation of these non infecting forms, the results present low consistent for drug development. However it is useful as initial screening test and also can provide some information about anti-trypanosomal action of tested compounds.

As showed from **figure 5**, no dose-response relationship could be observed at tested concentrations. Albeit a slight tendency of inhibition may be assumed, statistical analysis was not able to evident any difference between values obtained from different concentration of all compounds. However, when we analyze parasites growth inhibition as a function of time **(figure 6)**, joining together all results provided from any tested concentration, it is notorious some similarities concerning anti-trypanosomal action of the compounds. Compound TS9 presented an initial activity after 3 hours of exposition, and this lasted until 24 hours when epimastigota cells returns the proliferation indicating that TS9 exerts inhibition effect on the cellular growth rather than trypanocidal. By the other hand, benzonidazole and β-citronellol performed a continuous inhibition effect suggesting a trypanocidal activity followed an initial proliferative activity over the first three hours of exposition. Interestingly, the hybrid carbamate kept a mild anti-proliferative activity along the entire test, ranging about 92%, with a maximum activity of 81.36% (+/- 5,84%) at time 3 hours.

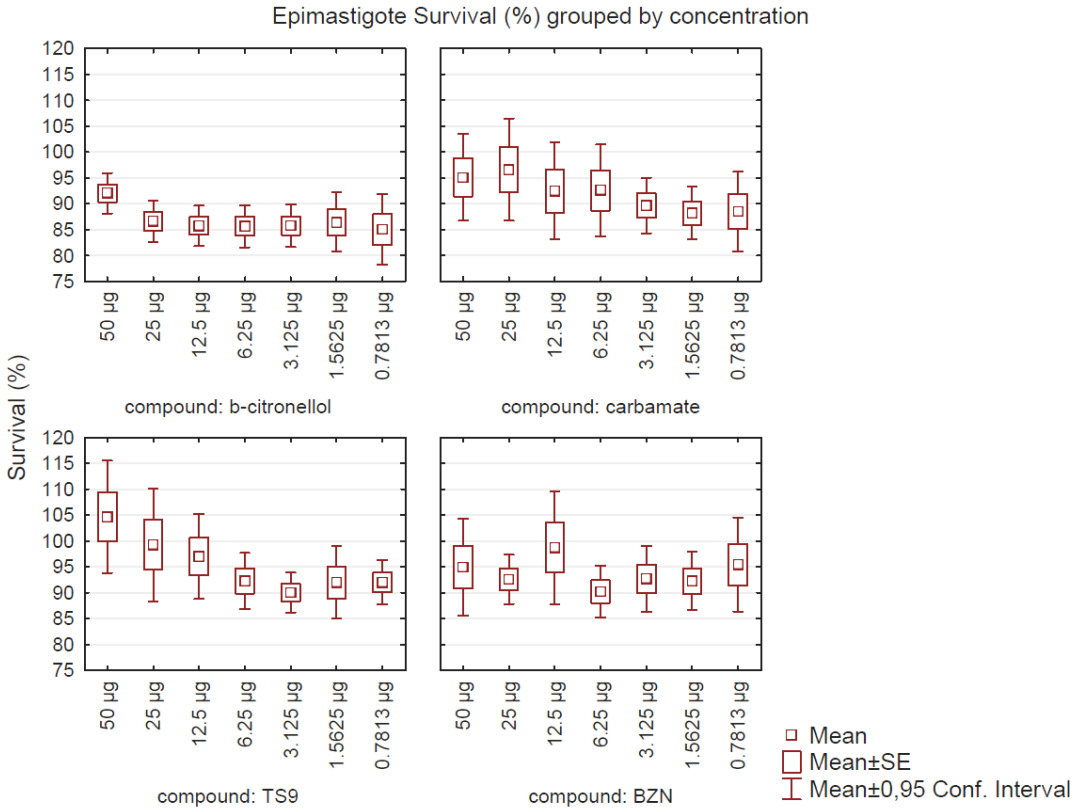


FIG. 5. Anti-proliferative effect of β-citronellol, hybrid carbamate, TS9 and benznidazole agaist *T. cruzi* epimastigote versus concentration (µg/500µL).

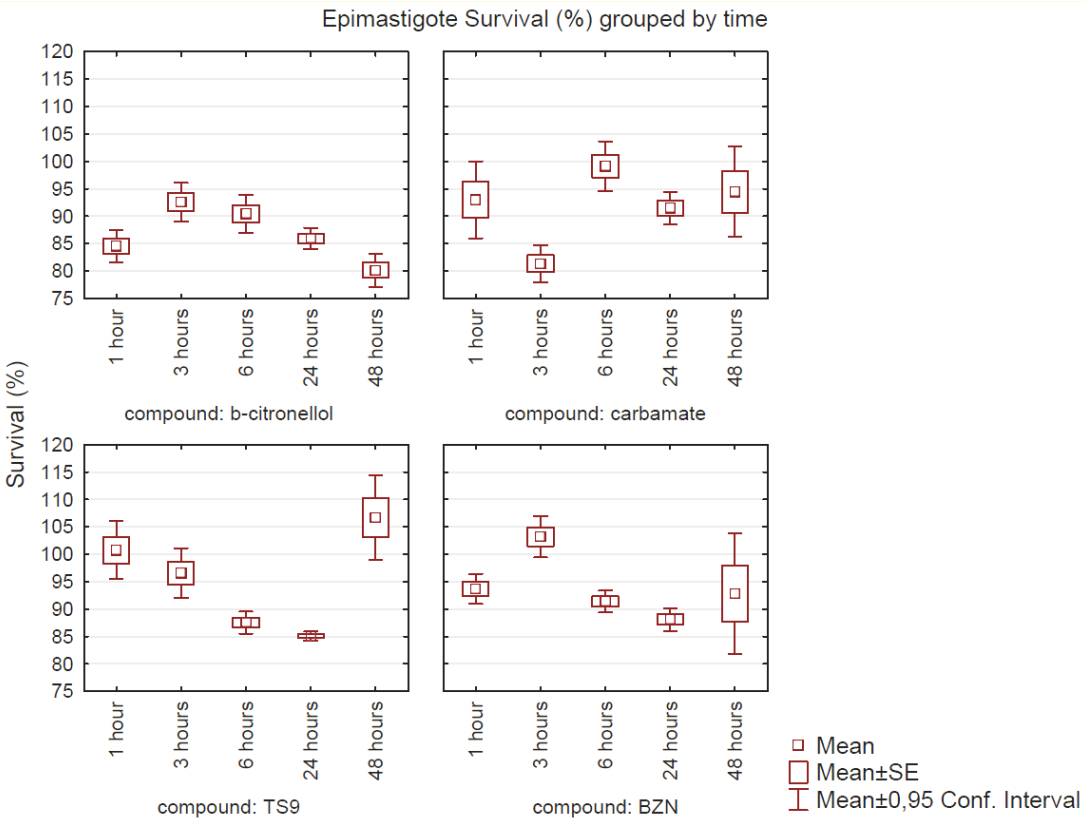


FIG. 6. Anti-proliferative effect of β-citronellol, hybrid carbamate, TS9 and benznidazole agaist *T. cruzi* epimastigote versus time.

Moreover, the overall average of inhibition for each compound, joining together all concentration at every time **(figure 7)**, show us that β-citronellol was the most effective tested compound (86.75%, +/- 6.75 SD; *P* = .003). No difference was observed between the average from benznidazole and TS9 or the hybrid carbamate (*P* > .05), but the hybrid (91.88%, +/- 11.11 SD) was slightly more effective than TS9 (95.31%, +/- 11.41 SD; *P* = .046).

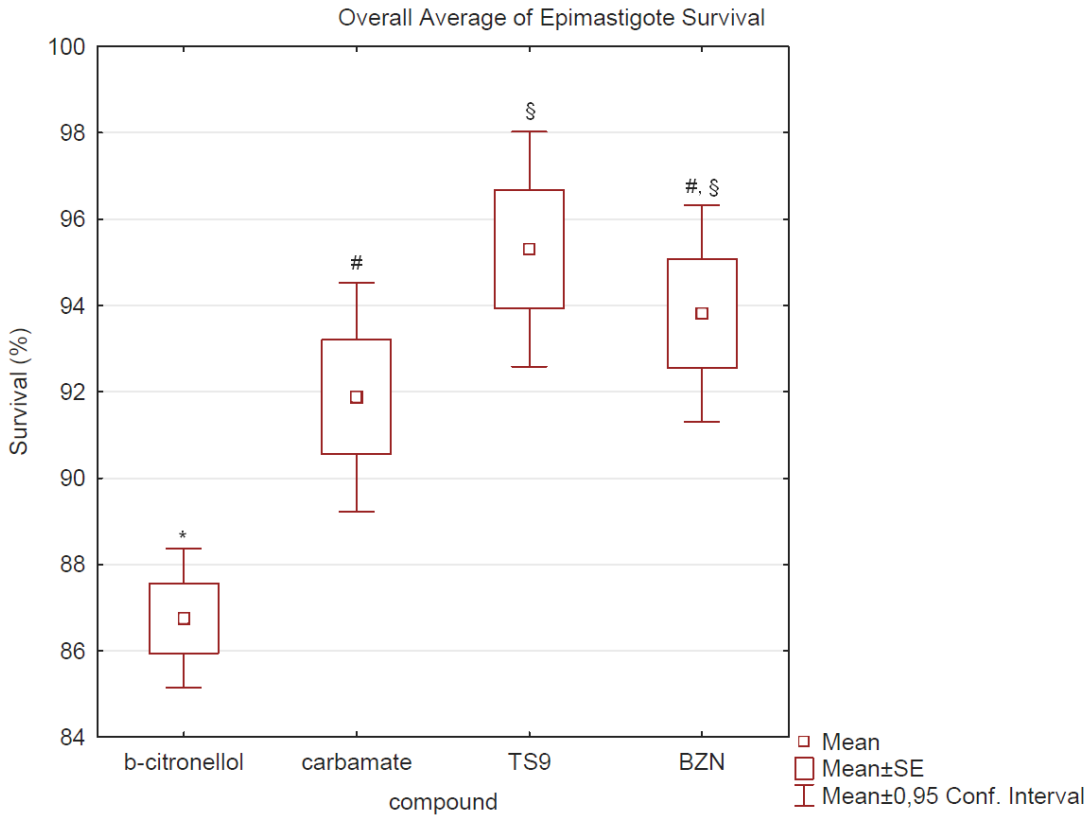


FIG. 7. Overall average of anti-proliferative effect against *T. cruzi* epimastigote forms.

Against trypomastigote forms of *T. cruzi*, no statistical difference could be observed between all tested groups, including the reference drug benzonidazole. In this test, where a short period of experimentation (2 hours of incubation) was planned to mimic biological condition concerning the lifecycle of trypomastigote in human blood, no tested compound was able to exert any antiproliferative action (**figure 8**). Possibly, prolonged time of incubation could lead to better results of trypanocidal activity, but these results are impossible to be reproduced at *in vivo* assays, once after this short time of existence on human blood, trypomastigotes must infect macrophages cell and differentiate to amastigote form. Even benznidazole (BZN), a reference drug for Chagas' disease treatment, was not able to significantly inhibit the grown of trypomastigotes during this time of experimentation. This observation is of extreme importance, once show us that benznidazole is ineffective against trypomastigote, acting specifically against amastigote, revealing the importance of trypanocidal studies against amastigote form of *T. cruzi*.

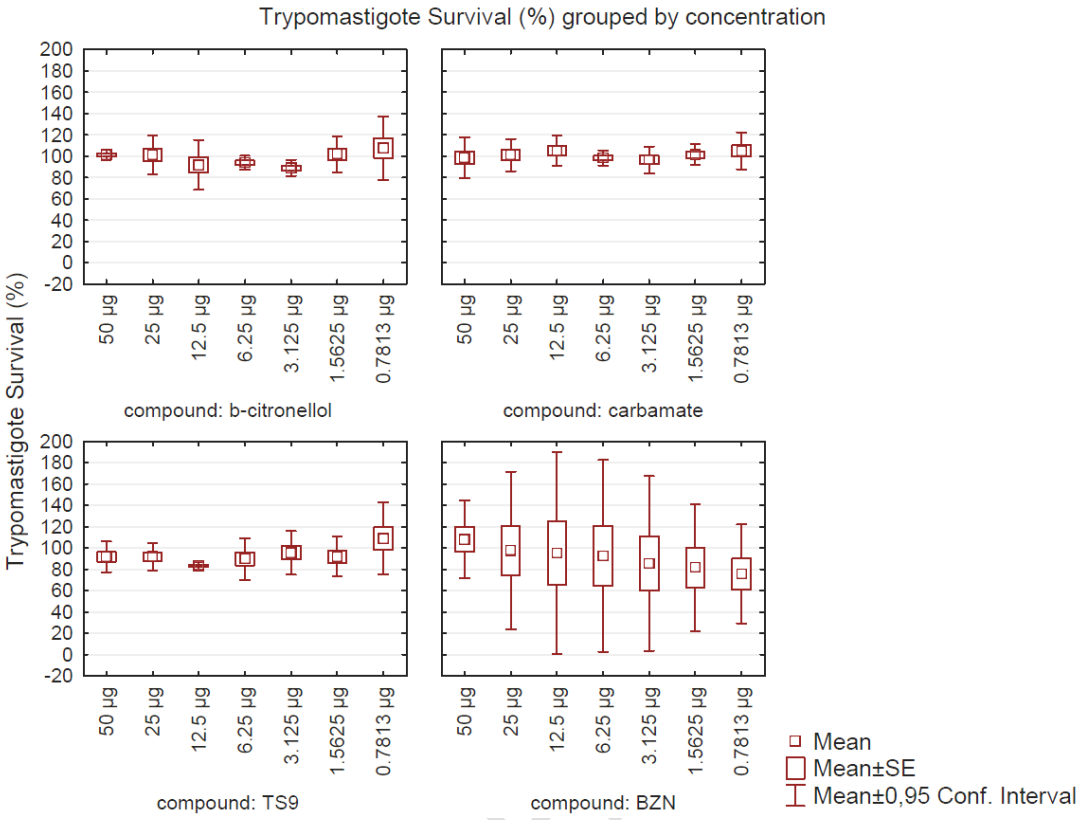


FIG. 8. Anti-proliferative effect of β-citronellol, hybrid carbamate, TS9 and benznidazole agaist *T. cruzi* trypomastigote versus concentration (µg/500µL).

So, the action of all tested compounds against amastigote deserves a special attention, and both effects over the number of infected macrophages cells and number of intracellular amastigote forms were considered in this study. By this way, the assay was performed in duplicate, observing at least 200 macrophages cells. No statistical difference could be observed on the totality of counted amastigote cells (P = .67) and the total number of infected macrophage cell (P = .13) between control groups of all treatment (when no drug was applied), indicating that the infection index was similar for every tested groups (average of 4,9%). So, the results were normalized to 100% based on the values reached for each control group.

For IC_{50} determinations, linear regression curves were obtained from the logarithm of concentration (µg/500µL), removing outliers pointed from a normal probability plot. All regression curves were submitted to a statistical T-test (α = .05).

From **figure 9**, where it is plotted the percentage of infected macrophage cell *versus* concentration of any tested compound, we can see that only the compound β-citronellol didn't showed a valid regression curve, indicating that this terpene doesn't affect the amastigote ability to invade macrophages, despite its trypanocidal action. By the other hand, both benznidazole and thiosemicarbazone derivatives showed activity in a dose-dependent way. This observation corroborates with the well known role of cruzipaine, the main target of thiosemicarbazones, which is involved in the penetration process and in the ability to infect and develop intracellularly in mammalian cells [19,20], whereas β-citronellol does not inhibit it.

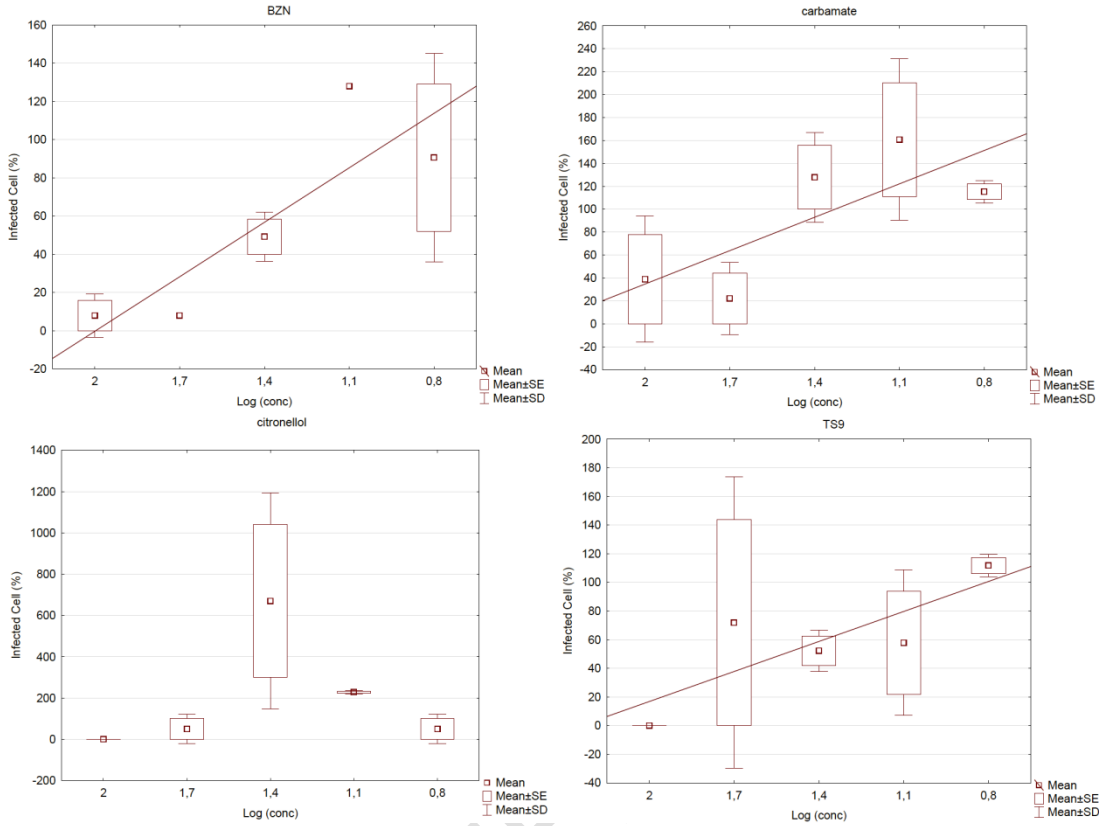


FIG. 9. Effect of β-citronellol, hybrid carbamate, TS9 and benznidazole over the number of infected macrophages cell versus logarithm of concentration (µg/500µL).

Trypanocidal activity of β-citronellol turns clear when the number of intracellular amastigote is observed **(figure 10 and table 2)**. TS9 was the most active compound with IC_{50} value of 44.73 µM (+/-0.1488) but its selective index (SI) was very poor (0.6) indicating that it is almost 2 times more toxic to macrophage cell than to parasites. The SI value was estimated as a ratio of the IC_{50} value against intracellular amastigote forms and the minimum non-toxic concentration to THP-1 cells. By the other hand, the best SI was reached for β-citronellol (SI = 28.5). Here we can see that the hybrid carbamate was able to lower TS9 toxicity but compromising its activity against amastigote cell. With an IC_{50} value of 146.93 µM, the hybrid showed better performance than the standard benznidazole (204.29 µM) and similar SI (3.9 against 4.7 for BZN). However, the overall analysis of amastigote activity of all compounds, the hybrid characteristic of the carbamate turns evident, and encourages us to pursue this design for further molecular modeling studies. The fact that β-citronellol seems not to interfere in the number of infected macrophage cell, but carbamate does interfere, added to the diminishing of cytotoxicity related to TS9, are evidences that the purpose of the hybrid design was successfully reached, keeping trypanocidal profile of both moieties (terpene and thiossemicarbazone), albeit it is notorious the necessity to improve its IC_{50} value and thus, the SI.

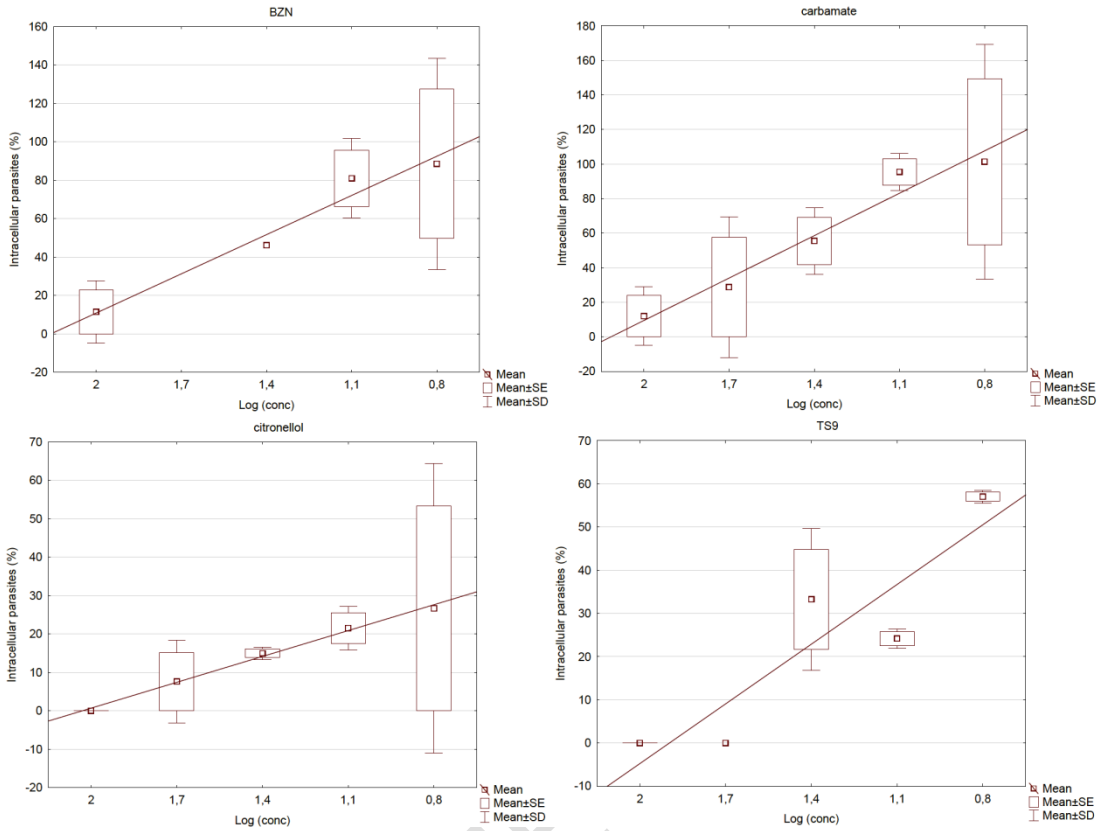


FIG. 10. Anti-proliferative effect of β-citronellol, hybrid carbamate, TS9 and benznidazole against intracellular *T. cruzi* amastigote versus logarithm of concentration (µg/500µL).

Table 2. Selective Index (SI) and IC_{50} values for trypanocidal action against *T. cruzi* amastigotes.

Compound	Linear regression (R^2)	IC_{50} (+/- SE)	SI
β-citronellol	y = -0,4023x + 0,7581 (0.8598)	56.07 µM (0.1324)	28.5
Hybrid carbamate	y = -0,8179x + 1,731 (0.9625)	146.93 µM (0.0685)	3.9
TS9	y = -0,4607x + 0,8739 (0.8229)	44.73 µM (0.1488)	0.6
benznidazole	y = -0,6806x + 1,4696 (0.9664)	204.29 µM (0.0648)	4.7

y=Log(concentration in µg/500µL), x=percentage of inhibition

Finally, the hybrid strategy can also provide another important feature for drug development. When we evaluate drug-likeness aspects based on Lipinski-rule of five, we can observe that choosing an adequate terpene moiety it is possible to improve such properties in order to preview an adequate oral absorption. From **table 3** we notice that there

were an increment of total hydrogen bond donors and acceptors in the hybrid carbamate comparing to β-citronellol, and also the high hydrophilicity of TS9 was balanced with a terpenic moiety, bring the value for a satisfactory LogP value of 3.63, while molecular mass and molar refractivity was kept into an appropriated range. This simple analysis shows us that the hybrid carbamate complies with the parameters of Lipinski-Rule of five, and may present better oral bioavailability profile than its precursors.

Table 3. Lipinski-rule of five analysis.

parameter	β-citronellol	TS9	hybrid carbamate
molecular mass	156.27	289.74	435.54
hydrogen bond donor	1	5	4
hydrogen bond acceptors	1	6	8
LogP	2.75	0.09	3.63
Molar Refractivity	49.53	68.61	121.35

Performed with MarvinSketch 18.11.0 software.

4. CONCLUSION

The strategy of hybridization of a terpenic compound and a synthetic thiosemicarbazone derivative shows a promissory way to develop new chemical entities, with satisfactory drug-like properties according to Lipinski rule-of-five. The hybrid was able to keep the trypanocidal features of both molecules employed, as planned. In addition, biological assays demonstrate that thiosecarbazone (TS9) cytotoxicity greatly decreased after hybridization, which able us to employ this very active compound for developing new analogues with better anti-proliferative effect against intracellular *T. cruzi* amastigotes. The selective index, however, was not satisfactory, although it was very similar to the one found for benznidazole, the reference drug for treatment of Chagas' disease.
So, the hybrid design was successful in the planned biological assays, and should be pursued for further molecular modeling studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

A1. Synthesis

A1.1. Preparation of 2-amino-1-(4-nitro) acetophenone thiosemicarbazone (TS9)

Compound TS9 was obtained as previously described by Blau *et al*. (2013). Structural identification was performed by comparison of the spectral data to the literature. **mp**: 173ºC; **IR** (KBr, cm^{-1}): 3491 – 2966, 1593, 1523, 1336, 1292, 1091; **^{1}H NMR** (DMSO-d6, 300 MHz, δ): 10.31 (s, 1H), 8.30 (m, 6H), 7.69 (m, 2H), 4,03 (br s, 2H). Reference Blau, *et al.* (2013): **mp**: 170-173 ºC; **IR** (KBr, cm^{-1}): 3487-2986, 1596, 1512, 1342, 1288, 1096; **^{1}H NMR**(DMSO-d6, 300 MHz, δ): 10.29 (s, 1H), 8.29-8.09 (m, 6H), 7.71-7.66 (m, 2H), 4.05-3.99 (br s, 2H).

A1.2. Synthesis of hybrid carbamate of β-citronellol and TS9

A mixture containing 3.3 mmol of β-citronellol, 0.03 mmol of DMAP and 5 mmol of *p*-nitrophenylchloroformate was prepared in 20 mL of THF at -10ºC. 5 mmols of 2,6-lutidine was dropwised keeping temperature between 0ºC and 5ºC. After 30 minutes, temperature was allowed to reach room temperature, about 25 ºC, and the reaction was monitoring by thin layer chromatography (TLC) for 24 hours along. The precipitant was filtered off and the remained solution was evaporated to dryness. A brown residue was treated with 25 mL of ethyl acetate, and washed with 0.1M HCl, saline solution and cold water. The organic phase was separated and dried, yielding 69% of the mixed carbonate of *p*-nitrophenol and β-citronellol.

The desired carbamate was synthesized from a 10 mL solution of TS9 (0.03 mmol in dichloromethane) containing 0.5 mL of *N,N*-diisopropylethylamine (DIPEA). 0.36 mmol of *p*-nitrophenol carbonate of β-citronellol was added over TS9 solution and the pH adjusted to 10 with DIPEA. The reaction was kept under nitrogen and protected from light for 6 hours, when no reaction with ninhydrin could be observed on TLC. The reaction mixture was dried under vacuum, and the residue purified by silica gel liquid chromatography, using dichloromethane and ethyl acetate (8:2) as mobile phase (53% yield).

Hybrid carbamate: **mp**: 110 ºC; **IR** (KBr, cm^{-1}): 3466-3350 (N-H), 3192-2926 (C-H), 1687 (C=O), 1589 (C=N), 1523 and 1340 (-NO_2), and 854 (C=S); **^{1}H NMR** (DMSO-*d6*, 300 MHz, δ): 10.78, 8.58, 8.22, 8.19, 8.18, 7.87, 5.04, 4.30, 4.00, 1.89, 1.62, 1.55, 1.54, 1.42, 1.32, 1.25, 1.10, 0.82.

A2. Spectral Data

A2.1. ^{1}H NMR spectral data of β-citronellol/TSC hybrid carbamate

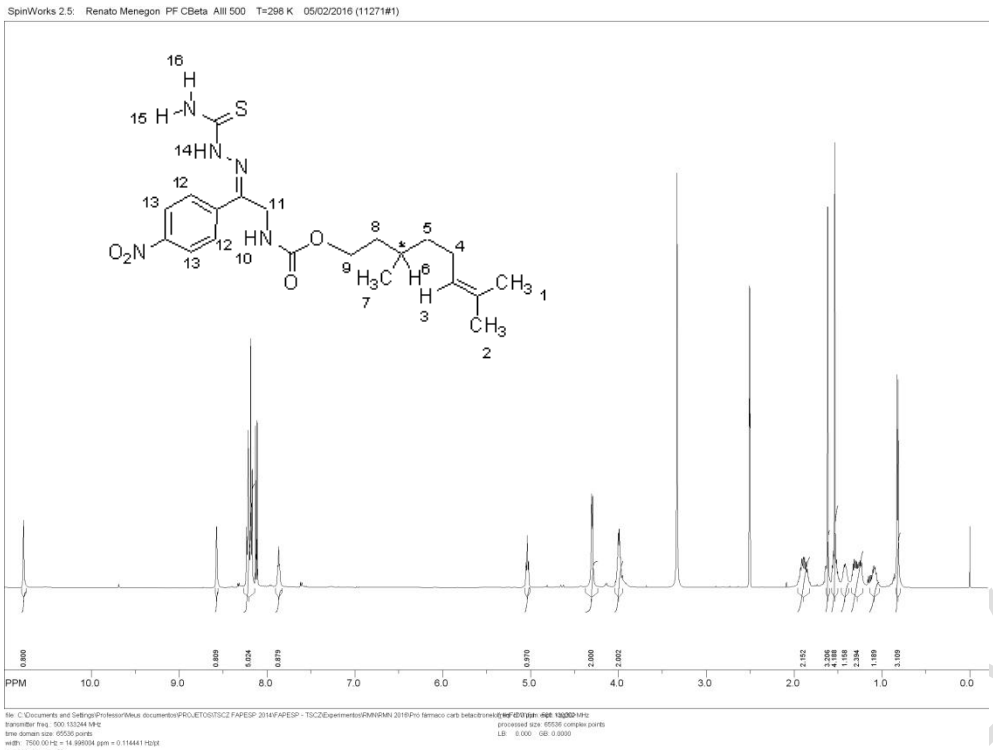


Fig. A1. ^{1}H NMR (300 MHz, DMSO-*d6*) spectra of hybrid carbamate

Position	δ ^{1}H (ppm)	gCOSY
1	3H 1.62 s	---
2	3H 1.54 s	5.04
3	1H 5.04 t (J=9.00, 7.00)	1.54; 1.89
4	2H 1.89 m	5.04; 1.25
5	1H a, 1.25 m 1H b, 1.10 m	5.04; 1.89 1.89; 1.25
6	1H 1.55 m	4.00; 1.32
7	3H 0.82 d (J=6.5)	1.42
8	1H a, 1.32 m 1H b, 1.42 m	4.00; 1.55 0.82
9	2H 4.00 m	1.55
10	1H 7.87 t (J=5.75, 7.9)	4.30
11	2H 4.30 d (J=5.75)	7.87
12	2H 8.18 m	---
13	2H 8.22 m	---
14	1H 10.78 s	8.58
15	1H 8.58 s	10.78; 8.19
16	1H 8.19 s	8.58

Table A1. ^{1}H NMR chemical shifts (ppm) of hybrid carbamate and two-dimensional ^{1}H-^{1}H correlations (COSY).

2.2. 2D COSY of β-citronellol/TSC hybrid carbamate

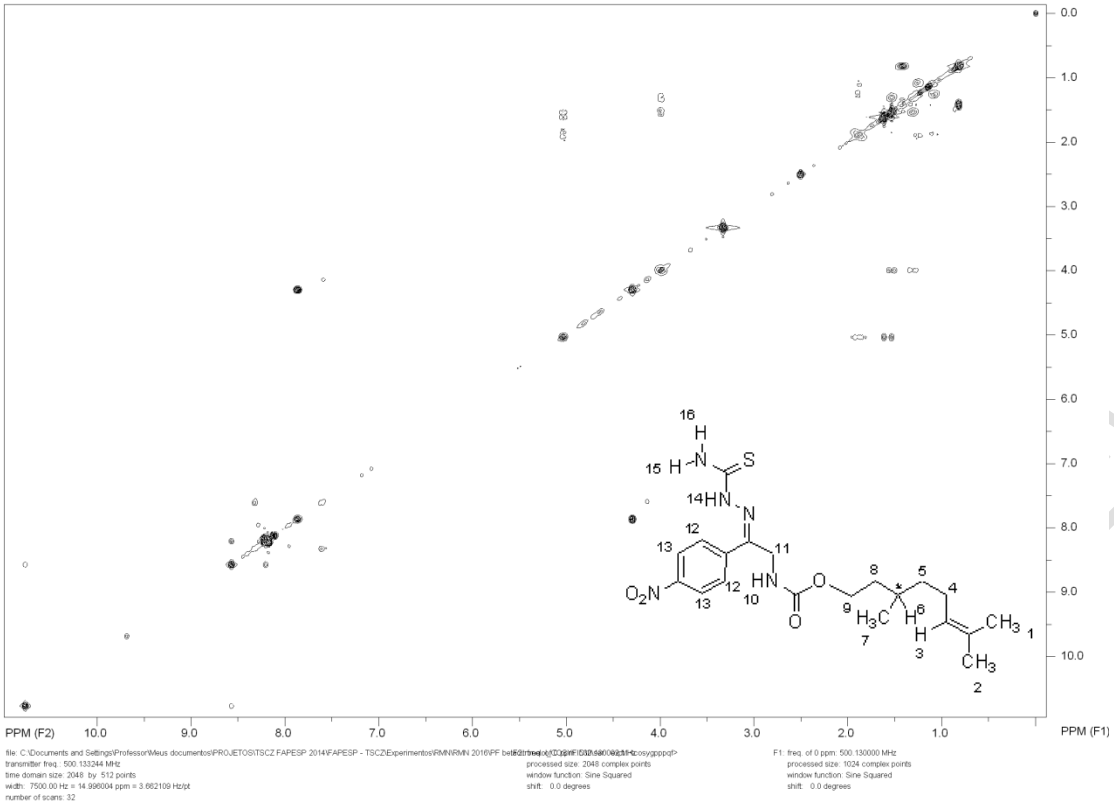
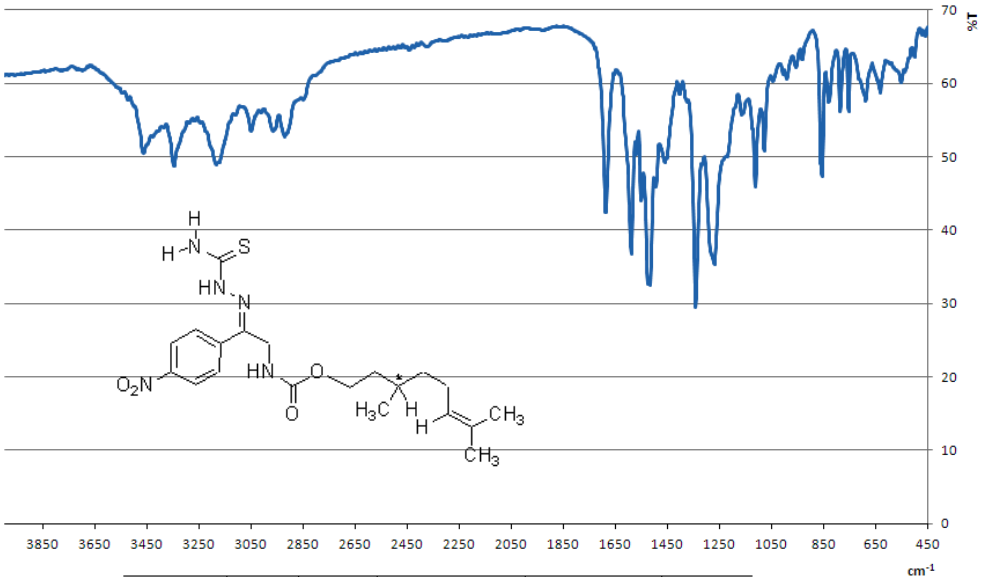


Fig. A2. ^{1}H COSY spectrum of hybrid carbamate (300 MHz, DMSO-*d6*).

A2.3. Infrared Spectrum data (KBr)



νN-H	νC-H	νC=O	νNO_2		νN-C-O		νC=S ax.
			Ax. Ass.	Ax. Sim.	Ax. Ass.	Ax. Sim.	
3466-3350	3192-2926	1687	1523	1340	1267	1112	854

554
555 **Fig. A3.** Infrared spectrum and frequencies (cm^{-1}) of hybrid carbamate (KBr)