

INFORM ABOUT THE EVALUATION OF HERBAL EXTRACT MIX AND OF ITS INDIVIDUAL COMPONENT ON CELLS BW5147 PROLIFERATION.

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I. ABSTRACT:

The anti-proliferative activity of a vegetal product was evaluated *in vitro* on murine lymphoma T, line BW5147. For it, studies were made dosage-answer to different times, in presence or absence of different extracts. The answer on the cellular proliferation was determined through the incorporation of thymidine triphosphate to the cellular DNA. The analyzed extracts were: 1 MT (mother tincture, composed by three individual tinctures: *Baccharis articulata*, *Rosmarinus officinalis* and major *Plantago*), 2 PF1 (dilution 1:10 of MT in water, containing 5 % v/v of alcohol), 3 PF3 (just as PF1 but with an alcohol concentration of 15 % v/v). All extracts, MT, PF1 and PF3, presented anti-proliferative activity on lymphoma cells. This effect was dependent of the concentration and was observed at 24, 48 and 72 hs of culture, reaching a maximum effect to the 48 hours of culture (Table I). Also was a synergic action between three individual tinctures, component of the extracts. The microscopic observation using a colorant of exclusion, demonstrated that MT and PF had a cytostatic activity at 24 hs of culture, without modifications of the cellular viability. Nevertheless, cytotoxic effects, were observed in greater culture periods (Table 2). These results were confirmed using another cellular line corresponding to lymphoma T derived from mice BALB/c, denominated LBC. Again, MT and PF presented anti-proliferative concentration and time action dependent on cells LBC. The maximum effect was reached to the 48 hours of culture (% of inhibition after MT: 1/500: 99± 0.5, 1/2000: 78.7± 0.6, 1/4000: 41± 0.3; for PF3: 1/50: 98.9± 0.8, 1/200: 89± 0.7, 1/400: 79± 0.5). Considering these results, can be concluded that both extracts MT and PF had an important anti-proliferative activity on the studied lines of lymphoma.

I. DESCRIPTION:

The anti-proliferative action was evaluated on lymphoma murine cells BW5147 *in vitro* by dosage-answer curves and at different times of exposition to these products, in cultures of them in presence and absence of this herbal extract, of its individual components or the vehicle. The proliferative answer was determined by the technique of [³H]-thymidine ([³H]-TdR) incorporation to cellular DNA and later evaluation of the nuclear radioactivity by spectrometry of liquid scintillation.

II. METHODOLOGY'S SPECIFICATION :

1- Herbal Extracts :

The herbal extracts evaluated correspond to the following description:

- 1- Mother Tincture (MT): Correspond to a tincture made by the mixture of three individual tinctures, to know: *Baccharis articulata*'s tincture 40 % v/v; *Rosmarinus officinalis*'s tincture 40 % v/v and *Plantago major*'s tincture 20 % v/v. The alcoholic content of mother tincture is of 50 %.

- 2- Final Product 1 (FP1): Correspond to a dilution 1/10 of mother tincture in water (Baccharis articulata's tincture 4 % v/v; Rosmarinus officinalis's tincture 4 % v/v , Plantago major's tincture 2 % v/v and alcoholic content of 5 % v/v).
- 3- Final Product 3 (PF3): Correspond to a dilution 1/10 of mother tincture, with an alcohol aggregate till a final concentration of 15 % v/v.

2- Lymphoma murine line:

The line used correspond to a lymphoma murine T, denominated BW5147, proceeding from a spontaneous tumor of mouse AKR/J adapted to culture, originated in the Jackson Laboratories, USA. The one is sensible to cortisol (10^{-6} M) and express the haplotype H-2^k and the following markers: CD3⁺, receptor T $\alpha\beta$ and Thy 1.1, these one are routinely checked by cytometry of flux with specific monoclonal antibodies.

3-Conditions of culture and evaluation of cellular proliferation:

The cells BW5147 ($3-5 \times 10^5$ cel/ml) were cultured in medium RPMI 1640 supplemented with 10% of bovine fetal serum and 2 mM of glutamine in presence of antibiotics penicillin (100U/ml) and streptomycin (100 μ g/ml), in plates of 96 cups (final volume 0.2 ml), in conditions of sterility (laminar flux, Sterilized Guard Hood class II, Type a/B3; mark: Baker Company, model: SG-400m), and in gasified ambient with 5% of CO₂ (gasified stove of CO₂; mark: Scientific form; model: 3111). It have been made cultures with increased concentrations of the herbal extracts already mentioned for 24, 48 and 72 hs of incubation. The cells were pulsed with 0.75 μ Ci/cup de [³H]TdR (S = 25 Ci/mmol) 16 hs before the sacrifice of cultures (by freezing at -20 °C).

Later the cultures were unfreezed and filtered by glass fiber's filters, Whatmann GF/A. The [³H]-TdR incorporated to nuclear DNA and stopped in these filters was quantified by spectrometry of liquid twinkle using cocktails of commercial twinkles. The results (obtained in dpm) were expressed as Percentage of inhibition (% Inhib) considering as 100% a the dpm obtained in absence of extract and/or in presence of vehicle.

4- Determination of cellular viability:

The cellular viability in different times in presence or absence of MT and FP was evaluated by microscopic cellular count in Neubauer's camera, using a colorant of exclusion, the Blue Tripan. The correspondents % of viability were calculated taking into account the quantity of alive cells (that don't include the exclusion colorant) with respect to the total cells (Alive + Death, just to say the cells that incorporate the colorant and are seen blue colored at microscopic observation).

5- Statistic analysis of results:

The results were analyzed by the Student's Test and Varianza Analysis followed by Dunnet's test to determine the significant differences between groups. The values were considered statistically significant when $p \leq 0.05$.

III. RESULTS

1. Action anti-proliferate dosage-answer of herbal extracts on cells BW5147 at 24 hs's culture:

TABLE 1: Percentage of proliferate inhibition of lymphoma T cells induced by increased concentrations of the product, of its mother tincture and of FP3, at 24 hs of culture.

DILUTION ^a	MT	DILUTION ^a	FP1	FP3
	% Inhib ^b		% Inhib ^b	% Inhib ^b
1/25	93.3 ± 2.9*	1/2.5	N.D.	N.D.
1/100	79.5 ± 5.9*	1/10	84.7 ± 3.6*	85.3 ± 3.9*
1/250	60.3 ± 2.5*	1/25	71.4 ± 3.1*	71.1 ± 7.7*
1/500	51.5 ± 3.4*	1/50	44.4 ± 5.2*	45.0 ± 4.0*
1/1000	30.8 ± 3.5*	1/100	23.7 ± 5.1*	49.0 ± 2.5*
1/2000	22.2 ± 3.7 [#]	1/200	17.0 ± 2.5 [#]	28.7 ± 2.4*
1/4000	10.7 ± 1.1	1/400	5.1 ± 0.9	22.7 ± 2.6 [#]
1/8000	6.0 ± 0.7	1/800	1.1 ± 0.8	5.2 ± 0.5

^a There were used correlatives dilutions of MT and FP according to its proportion in MT. The total volume of dilution of herbal products added was 0.02 ml, maximum volume that can be added without diluting the contribution of nutrients in the culture medium. It must be remarked that for this reason hasn't been evaluated (N.D. = not determined) the dilution FP1 and FP3 (1/2.5) is equivalent to dilution 1/25 of MT.

^b The percentages of inhibition were calculated considering as 100% the radioactivity of [³H]-TdR incorporated in basal cultures (to say, in absence of herbal product): 24024 ± 1206 dpm. It's remarkable that the presence of 1.5 and 2 % of alcoholic vehicle (alcoholic content of the dilution 1/25 of FP3 and of MT respectively) stimulates cellular proliferation in about 25 % . All the others dilutions of herbal extracts were made maintaining a constant final concentration of alcohol of 0.5% that didn't affect basal proliferation. The results showed are the average ± E.S. of n=5 experiments made by triplicate.

* Defers significantly from the basal with p ≤ 0.01

[#] Defers significantly from the basal with p ≤ 0.05

2. Action dosage-answer of individuals tinctures on the proliferation of BW5147 cells at 24 hs of culture:

TABLE 2: Effect of increased concentrations of individuals tinctures on BW5147 cells.

DILUTION ^a	PLANTAGO (P)	CARQUEJA (C)	ROMERO (R)
	% Inhib ^b	% Inhib ^b	% Inhib ^b
1/25	26.0 ± 0.8 [#]	56.8 ± 6.5*	92.0 ± 2.8*
1/50	13.8 ± 8.6	38.5 ± 2.5*	85.8 ± 5.1*
1/100	1.5 ± 0.3	24.8 ± 7.2	70.0 ± 8.5*
1/500	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1

^a Each extract of individual tincture was diluted in culture's medium containing a final concentration of alcohol of 50% and in the same proportions that are contained in MT. In the plate was made a positive control correspondent to the dilution 1/25 of MT with the one was obtained in all the cases a % Inhib \geq 94%.

^b The percentages of inhibition showed are the media \pm ES of n=2 determinations made by triplicate and were calculated as was explained previously.

* Defers significantly from the basal with $p \leq 0.01$

Defers significantly from the basal with $p \leq 0.05$

3. Action anti-proliferate dosage-answer of herbal extracts on BW5147 cells at 48 hs of culture:

TABLE 3: Percentage of inhibition of lymphoma T cells induced by increased concentrations of the product, of mother tincture and FP3, at 48 hs of culture

DILUTION ^a	MT	DILUTION ^a	FP1	FP3
	% Inhib ^b		% Inhib ^b	% Inhib ^b
1/25	99.4 ± 0.1*	1/2.5	N.D.	N.D.
1/100	99.4 ± 0.4*	1/10	98.8 ± 0.9*	99.5 ± 8.0*
1/250	91.2 ± 0.8*	1/25	97.4 ± 6.4*	98.8 ± 7.0*
1/500	80.0 ± 0.5*	1/50	86.4 ± 2.7*	87.5 ± 6.0*
1/1000	38.8 ± 0.9*	1/100	31.0 ± 0.4*	73.7 ± 1.2*
1/2000	25.1 ± 1.1*	1/200	13.8 ± 1.1 [#]	30.6 ± 2.0*
1/4000	9.7 ± 0.2	1/400	11.5 ± 3.8	24.0 ± 2.1*

^a There were used correlatives dilutions of MT and FP as was described in Table 1.

^b The percentages of inhibition were calculated taking as 100% the radioactivity of [³H]-TdR incorporated in basal cultures: 32360 ± 1165 dpm. At 48 hs of culture weren't observed significant differences with con 1.5% and 2 % of alcoholic vehicle (dilution 1/25 of FP3 and MT, respectively) respect to basal proliferation. All the other dilutions of herbal extract were made maintaining a constant final concentration of alcohol of 0.5% that didn't affect the basal proliferation. The results showed are the average ± E.S. of n=5 experiments made by triplicate.

* Defers significantly from the basal with p ≤ 0.01

[#] Defers significantly from the basal with p ≤ 0.05

4. Action anti-proliferate dosage-answer of herbal extracts on BW5147 cells at 72 hs of culture:

TABLE 4: Percentage of inhibition dosage-answer induced by the product, its mother tincture and FP3 on the proliferation of lymphoma T cells at 72 hs of culture.

DILUTION ^a	MT	DILUTION ^a	FP1	FP3
	% Inhib ^b		% Inhib ^b	% Inhib ^b
1/500	54.1 ± 6.8*	1/50	61.2 ± 6.8*	64.5 ± 5.1*
1/1000	49.0 ± 4.0*	1/100	40.1 ± 2.8*	46.0 ± 4.6*
1/2000	39.3 ± 3.1*	1/200	20.6 ± 1.1 [#]	26.1 ± 3.0*
1/4000	29.0 ± 4.8 [#]	1/400	19.4 ± 1.7	28.8 ± 1.7*

^a There were used the correlatives dilutions of MT and FP as was described previously.

^b The percentages of inhibition were calculated taking as 100% the radioactivity of [³H]-TdR incorporated in basal cultures: (16781 ± 1188) dpm. In all the dilutions was maintained a 0.5% of alcoholic content that didn't modified the basal proliferation.

The results shown are the average ± E.S. de n=5 experiments made by triplicate.* Defers significantly from the basal with p ≤ 0.01[#]. Defers significantly from the basal with p ≤ 0.05

5. Action of herbal extracts on the viability of BW5147 cells at different times of culture:

TABLE 5: Effect of MT and FP3 on the viability of cultures cells at different times of the study

TIME (hours)	% VIABILITY ^a					
	MT			FP3		
	1/500	1/2000	1/4000	1/50	1/200	1/400
24	61.3 ± 10.9 [#]	84.7 ± 5.1	71.7 ± 2.0	72.5 ± 11.4	72.0 ± 6.6	85.6 ± 4.5
48	50.3 ± 11.3*	80.3 ± 4.1	76.7 ± 0.6	49.0 ± 9.8*	77.0 ± 3.3	76.7 ± 2.9
72	34.3 ± 1.0*	76.7 ± 1.9*	84.3 ± 2.8 [#]	24.3 ± 1.2*	60.4 ± 2.3*	80.6 ± 2.8*

^a The % of viability at each time (average ± ES of n=3 experiments) were calculated taking into account the quantity of alive cells (that not include the exclusion colorant Blue Tripán) respect to the total cells (alive + death: cells that incorporate the colorant and are seen of color blue at microscopic observation). The results are compared with the averaged values correspondent to the basal and to the incubated cells by the same times in average with 0.5% of alcohol, which are detailed at continuation: (95.3 ± 1.2) %, (94.0 ± 1.4) % and (94.3 ± 1.0)% at 24, 48 and 72 hs, respectively.

* Defers significantly from the basal values with $p \leq 0.01$

[#] Defers significantly from the basal values with $p \leq 0.05$

CONCLUSIONS:

- 1- It's observed an inhibitory effect (Fig. 1-3, Tables1, 3 y 4) of the proliferation of lymphoma T murine BW5147 cells induced by herbal products MT, FP1 and FP3. This effect is concentration dependent. It's observed in the 3 times studied, 24, 48 and 72 hs of culture. It's maximum at 48 hs of culture to the highest dosage and it's seen a potency action at 72 hs of culture of the effects induced by the bigger dilutions. In this time it must be considered however the possible contribution to inhibition mechanisms by contact between the cells in culture, that duplicate every 12-14 hs (Cremaschi et al, J. Neuroimmunol, 2000, 110: 57).
- 2- It's been found concordance between the effects exercised by FP and MT, in the three times studied.
- 3- The evaluation of the effects of the extracts of individual tinctures diluted and tested in the same proportions that the ones contained in MT, permit to verify a synergic effect of the three components (Table 2, Fig. 4).
- 4- The microscopic observation with an exclusion colorant permit infer that also FP as MT has a cytostatic effect at 24 hs that didn't modified significantly cellular viability, however with the time it seems to occur a cytotoxic effect that can't be discharge the participation of inherent factors to cellular culture (Table 5, Fig. 5 y 6).

Final Conclusion: Also MT as its FP exercise an anti proliferate effect overwhelming on lymphoma T BW5147 that justify to prosecute with the evaluation of their action on the biological behavior in lymphoma.

Figure 1: Percentage of inhibition of the proliferation of lymphoma T cells dosage-answer induced by mother tincture at different times of culture.

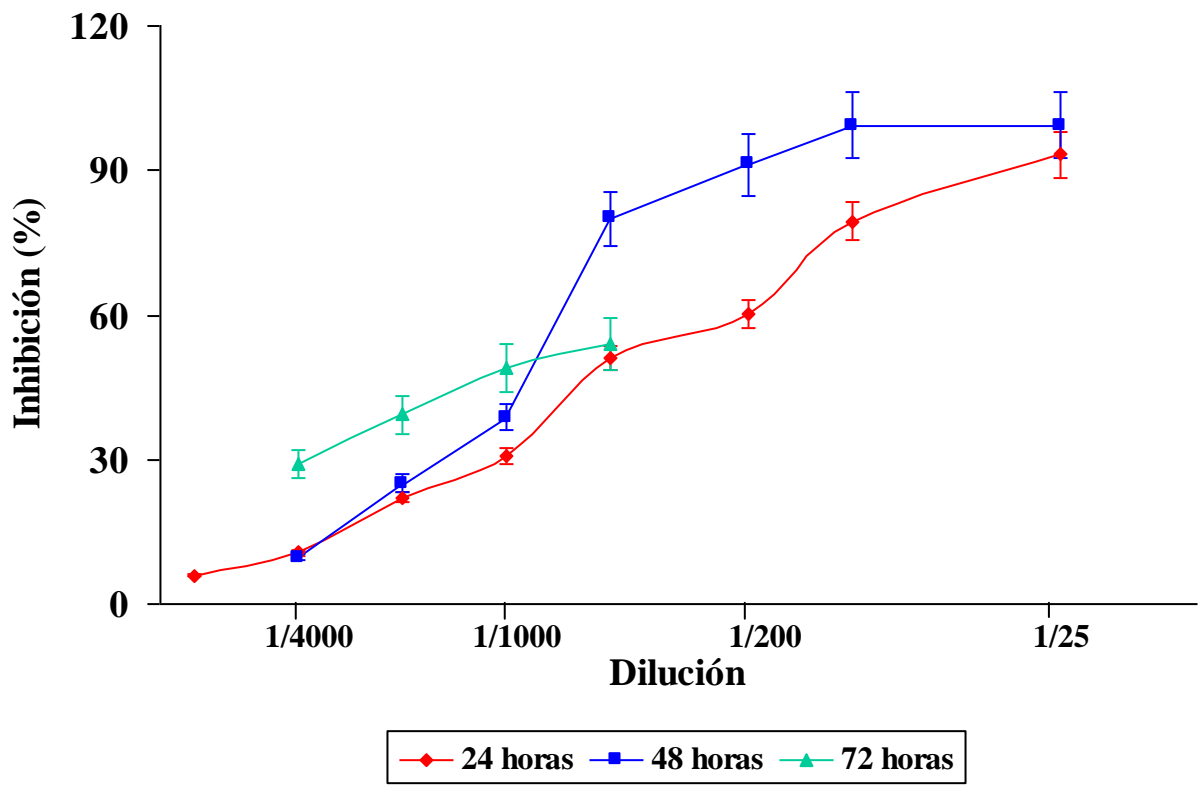


Figure 2: Percentage of inhibition of lymphoma T cell's proliferation dosage-answer induced by final product 1 (FP1) at different times of culture.

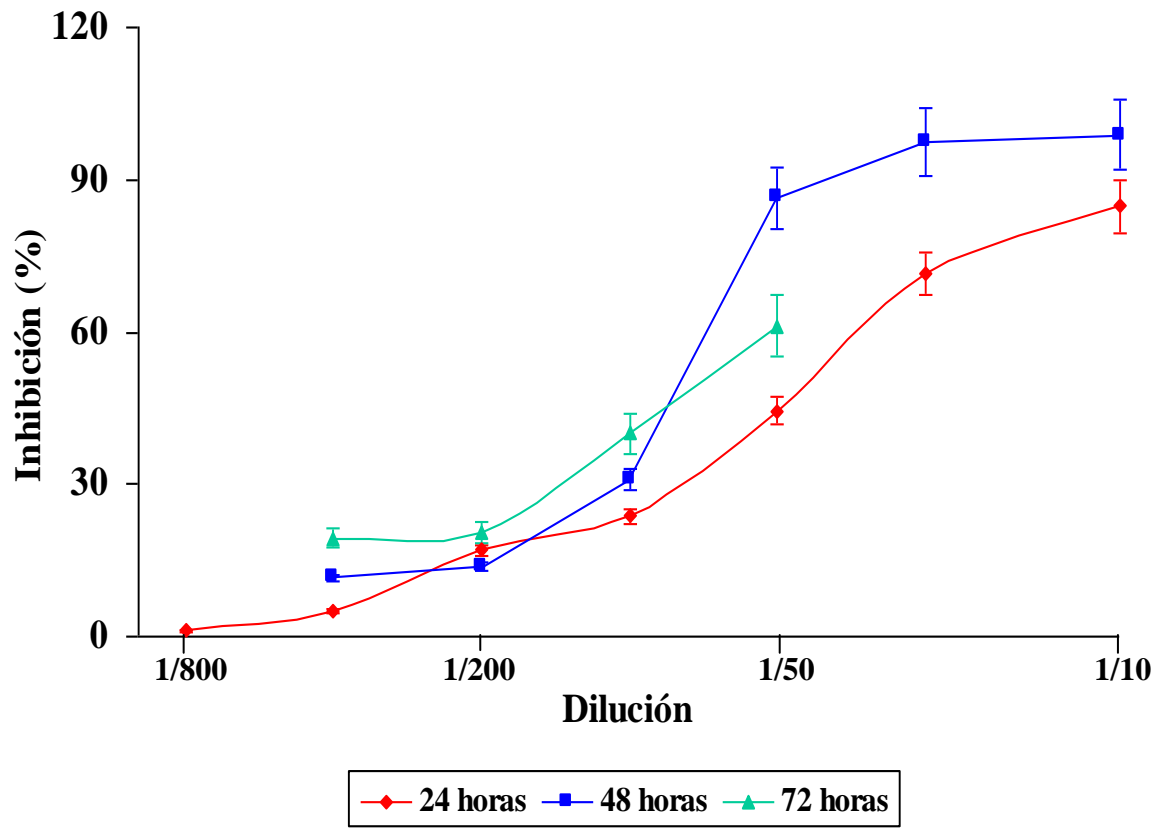


Figure 3: Percentage of inhibition of lymphoma cell's proliferation dosage-answer induced by final product 3 (FP3) at different times of culture.

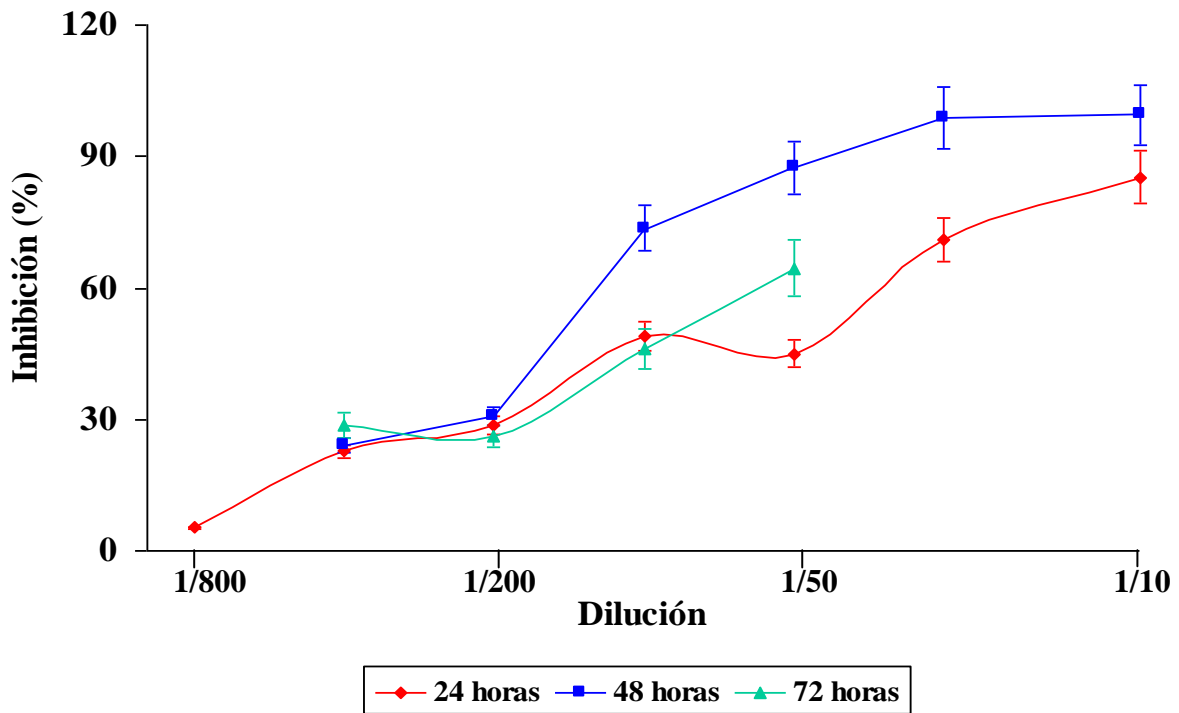


Figure 4: Percentages of inhibition of BW5147 cell's proliferation induced by individuals tinctures.

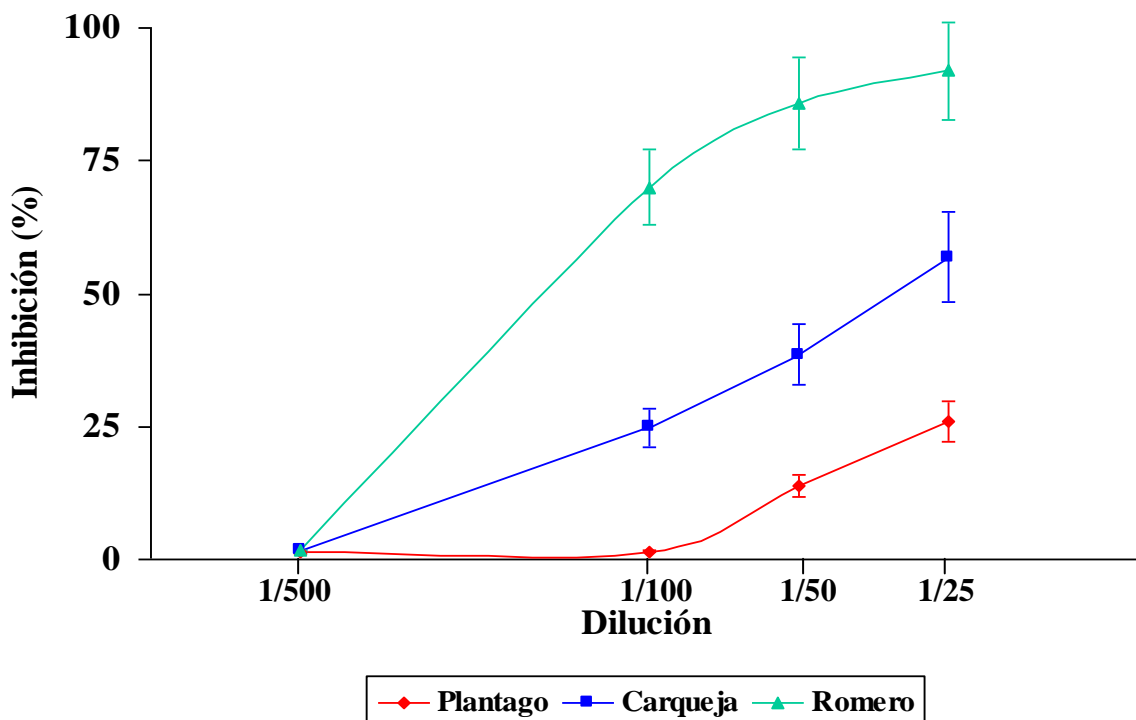


Figure 5: Effect of mother tincture on the viability of BW5147 cells at different times of culture.

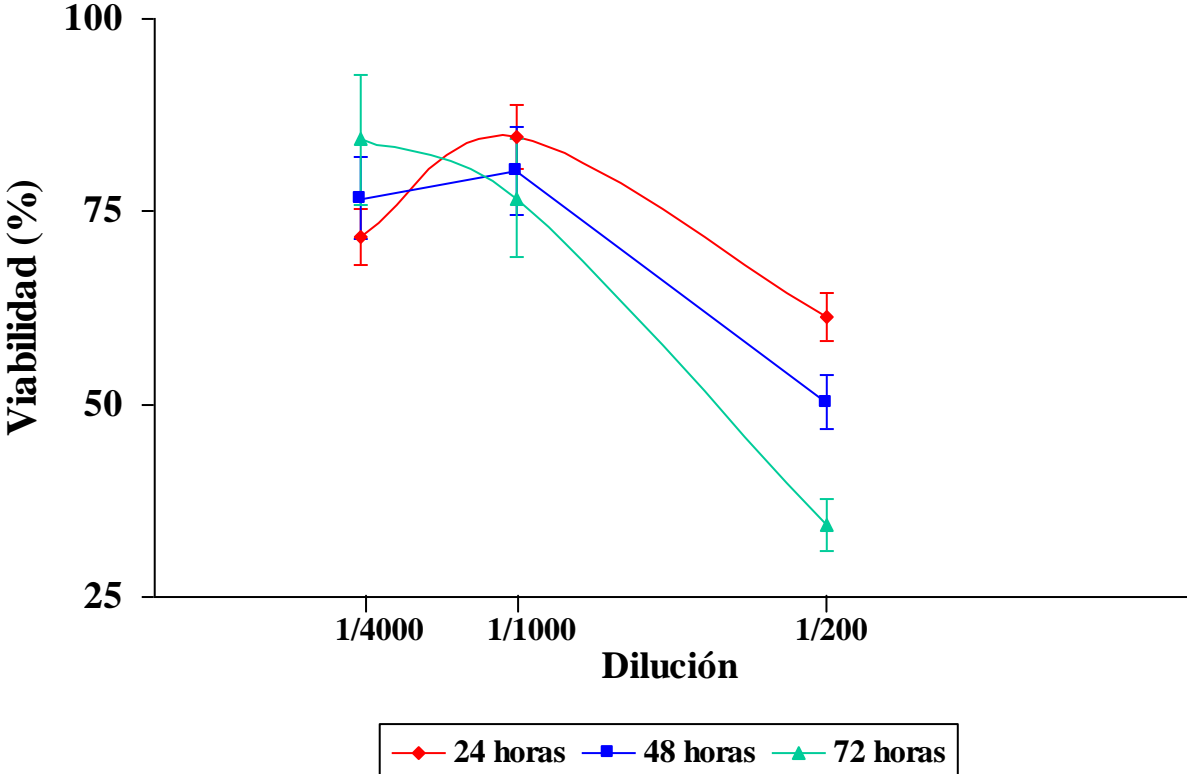


Figure 6: Effect of final product 3 (FP3) on the viability of BW5147 cells at different times of culture.

