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Extract-based microemulsion formulation with *Solanum aethiopicum* peduncle: characterization by DLS, relative humidity and F0 anti-proliferative effect on leukemia cancer cells

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Abstract
The appropriated type of formulation for an efficient bioavailability of phytocompounds (PCD) and plant extracts still a huge challenge for scientists and pharmaceutical industries as extracts generally contain multiple of hydrophobic and hydrophilic substances with different solubility. Even though the richness of *Solanum aethiopicum* L. (Solanaceae) in bioactive substances has already been reported, never the crude extract (F0) has been used in a formulation in order to assess the best way of administration of the embedded phytochemicals. The aim of this work is firstly to manufacture an extract-based microemulsion (µEm Labrasol-F0), characterize its stability and secondly, to evaluate the anticancer activity of F0 on Jurkat cells. Dynamic light scattering (DLS) results show that the particles size distribution in F1-F4 is ranged from 128 ± 14.6 nm (0.1 µm) to 3862 ± 772 nm (4 µm). Hygroscopic values in 97 % K_2SO_4 RH attest a better stability of µEm Labrasol-F0, F0- F4 moreover, F0 has anti-proliferative effect against Leukemia cancer cells.

Keywords: Microemulsion formulation, surfactant, stability, safeness, phytochemicals

1. Introduction
Growing From one of its primary definition, a microemulsion (µemulsion) is a system of water, oil and an amphiphilic which is a single optically isotropic and thermodynamically stable liquid solution it is either oil-in-water (O/W) or water-in-oil (W/O) according to the amount of the dispersed or continuous phase [1]. The associated change of energy for µemulsion formation is expressed by the following expression [2]:

$$\Delta G\, form = \Delta A \gamma_{12} - \Delta TS\, conf \quad (1)$$

Where; ΔG form (Gibbs free energy of formation), ΔA is the change in interfacial area A (equal to $4\pi r^2$ per droplet of radius r) and γ_{12} is the interfacial tension between phases 1 and 2 at temperature T (Kelvin). One of the most used equation for determining HLB values is Davies method which as the advantage of taking into account the effect of stronger and weaker hydrophilic groups [3]:

$$HLB = 7 + \sum_{i=1}^{m} H_i - n \times 0.475 \quad (2)$$

Where: *m* is the number of hydrophilic groups in the molecule, *Hi: The* value of the i^{th} hydrophilic groups and *n* the number of lipophilic groups in the molecule. The droplets dispersed in the continuous medium are smaller in µemulsions and ranged from 10 to 200 nm while sizes from 1 to 20 µm are found in conventional emulsions and it is likely that these types of formulations can be used as appropriate drug delivery systems and good drug carriers [4-6]. Two µemulsions were used in this work and their stability assessed compared to F0-F4 extracts.

2. Materials and methods
2.1 Material
Labrasol; code: 3074JV1 was supplied by Gattefossé (Canada), Isopropyl myristate (98%, cas: 110-27-0, Sigma Aldrich, Malaysia), Plurol oleique CC97 CG (cas: 9007-48-J purchased from Bionord AB, France), Polyethylene Glycol 400 was delivered by Sigma Aldrich, Sweden. A balance type Mettler PE 3600, Delta Range, Käglinge Vågtjänst AB (Switzerland) used and K_2SO_4 (cas: 7778-80-5 purchased from Sigma Aldrich, Sweden. An optical microscope model CKX41SF, Olympus Optical Co. LTD, Japon was used.

2.2 Methods

2.2.1 Formulation of extract (F0)-based microemulsion

The plant material and the crude extract F0 preparation have been already reported [7]. The formulation of the µemulsion "Labrasol-F0" (µEm Labrasol-F0) was prepared stepwise based on modified protocol containing the crude extract F0 and Labrasol as mean surfactant [8]. In a clean, sterile jar, an adequate amount of Polyglyceryl-3 oleate and Polyethylene Glycol 400 (wt%, 1:1) were mixed under constant magnetic stirring at 50 rpm with Caprylcaproyl macrogel glycerides (0.34:1) and 83 mg/ml of the extract F0 used as active substance, Isopropyl myristate (0.7:1) and (1:2) Milli Q water added dropwise. The second µemulsion is used in prevention of allergic rhinitis [9]; µEm T-F0 is a µemulsion containing no API to which 83 mg/ml of F0 were dissolved as mean active substance to have the so called µEm T-F0. Both preparations were made at room temperature, 4 ml of each perfectly formed µemulsion (without air bubbles, transparent) were ultra-sonicated by an ultrasonic cleaner for 5-10 min and the solutions stored at – 4 °C for stability analysis and different studies.

2.2.2 Characterization of particle size distribution by Dynamic light scattering (DLS)

DLS is a powerful analytical technique used to determine the size distribution of small particles in a suspension, the fractions of F0 (F1-F4) were used for more accuracy in the measurement in this analysis. An aqueous extract (10 µL) of F1-F4 was introduced into a micro cuvette and placed in the DLS spectroscope (type Particle sizer Nicomp 380 ZLS Systems/ Zeta potential, Santa Barbara, California, USA). The analysis time, 10 min for each sample; the size of dispersed particles in the sample is automatically calculated and displayed as a histogram; where the number of histogram for a particular sample characterizes the group of particle or molecule in the suspension are described by their mean diameter and percentage of distribution.

2.3 Hygroscopic strength of F0-F4 and microemulsions by Relative Humidity (RH)

In order to characterize the ability of the extracts and µemulsions to uptake water from their surrounding environment, a known mass of each sample was placed in 97% (m/v) relative humidity caused by silica gel and saturated K_2SO_4 solution in a closed glassware dessicator. The samples were weighted at different time points for 259 h to evaluate the water uptake by the samples over time until a constant weight was observed. The observed constant weight characterizes the maximum of water absorbed in each sample then the percentage of water uptake calculated and compared for each sample [10, 11].

2.4 Cell culturing and microscopic analysis of Jurkat cells upon influence of F0

Cell culturing according to reported procedure [7]. After hemocytometer counting, $0.5x10^6$ viable Jurkat cells were used in 96 wells plates round bottom (Sarstedt inc. USA) and treated with 25 mg/ml of F0 in RPMI medium, triple replicates including control cells were performed for 24 and 48 h and the effect of F0 on the cells analyzed and pictured by an optical microscopy [12].

2.5 Statistical analysis

The experiments were repeated three times, the results averaged and compared to controls. $\alpha = 0.05$ was used as theoretical value and Student's t-test was performed; the differences were considered significant between compared mean values when the probability (P) associated with the statistic is $P < \alpha$ and not significant for $P \geq \alpha$.

3. Results and Discussion

As outcome of triple replicate experiments, the proposed extract-based µemulsion formulation pattern using F0 as active substance is describe in Table 1 including the suitable components and their characteristics. The ratio of water / oil in the formulated Labrasol-F0 is about 10.

Table 1: Ingredients of used for F0-based µemulsion (Labrasol-F0, O/W) formulation

Compound's name	Wt%	HLB	Description
Plurol oleique (Polyglyceryl-3 oleate)	1 :1	6	Polar lipid (co-surfactant 1)
Polyethylene Glycol 400	1 :1	11.6	Polar solvent (co-surfactant 2)
Labrasol (Caprylcaproyl macrogel glycérides)	0.34 : 1	12	Polar solvent (surfactant)
Isopropyl myristate	0.7 :1	11.5	Oil phase (non-polar solvent)
Milli Q water	1 : 2		Water phase
F0	(83 mg/ml)		Active substance

O/W: formulation oil in water
HLB: Hydrophilic-lipophilic balance

Naturally, F0 may be substituted by any other extract or PCD and that will involve some adjustments of phases in order to obtain a perfect µemulsion (Figure 1). Extracts or PCD have different physicochemical properties; the solubility level, partition coefficient, sizes of the dispersed phase need to be considered, like F0; an extract could contain both hydrophilic and hydrophobic phytochemicals [13, 7]. The formulated µemulsion Labrasol-F0 has been simultaneously compared with another µemulsion [9] which is a commercialized product with no API used for nasal application, for comparison; the same concentration of F0 in Labrasol-F0 was added to µemulsion T as active substance and named µemulsion T-F0. Images below show the transparency of both µemulsions containing F0.

Fig 1: Images of A : µEmulsion T-F0 (W/O) et B : µEmulsion Labrasol-F0 ; (O/W)

Volume of each µemulsion = 4 ml, *Magnetic stir bars at the bottom for mixing (white), yellowish coloration: color of F0*
Both µemulsions are clear and isotropic solutions described [8]. as perfect µemulsions, apparently, µEmulsion Labrasol-F0 appears to be clearer than µEmulsion T-F0 but less viscous. The high viscosity of µEmulsion T-F0 can be explained by the fact that amongst the ingredients, µEmulsion T contains 24.3 % of Propylene glycol compared to µEmulsion Labrasol-F0 which does not contain Propylene glycol; this is in perfect agreement with the results of [6] who reported Propylene glycol as drug solubilizer, vehicle and nasal permeability enhancer for phytocompounds (PCD) and plant extracts. A pseudo-ternary phase diagram of µEmulsion Labrasol-F0 was drawn up based on the amount of the components used in the formulation [14, 15]. Figure 2 gives the details of the diagram with a displayed region (red point) of Labrasol-F0 µemulsification.

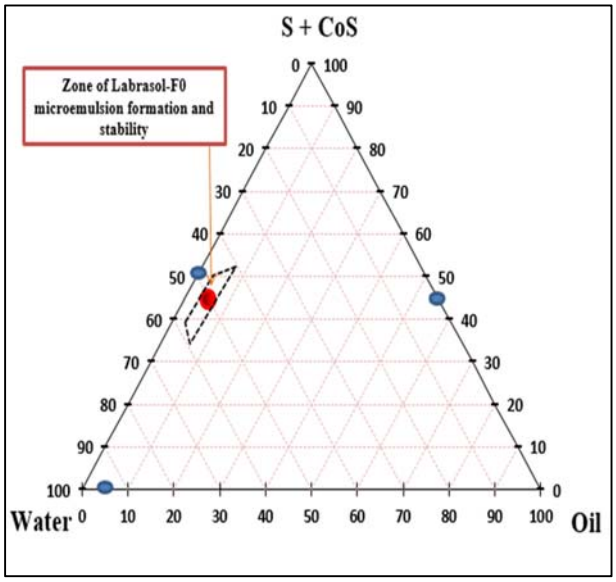


Fig 2: *pseudo*-ternary phase diagram of Labrasol-F0 µemulsion (O/W) *S + CoS: Surfactant + co-surfactants*

At this particular point (in red), the different concentrations of the ingredients used give the best, more stable and transparent formulation (Figure 1B) as described by [4]. The formulation is transparent and optically isotropic, it should be noted that more experiments performed outside this zone (red point) have given cloudy and unstable solutions. The surfactant (Labrasol) used in this formulation has a primary function to reduce or break the interfacial tension between water molecules and the oil phase for inducing the µemulsification which is possible in negative values of Gibbs energy of formation[2]. The type of surfactant used can affect the overall process, Labrasol with an HLB of 12 is appropriate for O/W µemulsion and the results confirm those [16]. who observed that surfactant of HLB > 10 could be better for µemulsion formulation. For our understanding; F0 is the mean extract which was fractioned into F1-F4 and analyzing the fractions give better details on the particle size distribution in F0, a particular interest is given to Labrasol-F0 as manufactured stepwise in this work. Labrasol-F0 is an O/W µemulsion containing F0 and water as continuous phase while the dispersed oil phase is Isopropyl myristate. DLS analyses have led to only find out the mean diameter of particles in suspension in F1-F4 but also notify the particles size range in F0, the outcome of DLS analyses are described by Figure 3A-D.

Fig 3A: DLS analysis: Particles size distribution in F1 (aq) of F0 *aq: aqueous extract, A: F1, B: F2, C: F3, D: F4, particles mean Diameter (mD) ± SD*

The aqueous solution of the fraction F1 of F0 (Figure 3A) shows from this result that; one type of particle are distributed in the solution represented by a single histogram and the average size of these particles (100 %) in the suspension is 1 µm. Figure 3B for instance describe the DLS result of F2 and it is clear that this fraction contains two types of particles in the suspension.
The average size of particle distributed in F2 is ranged from 0.1 to 0.4 micrometer and compared to those in F1, the particles in F2 are smaller in size (Figure 3B).

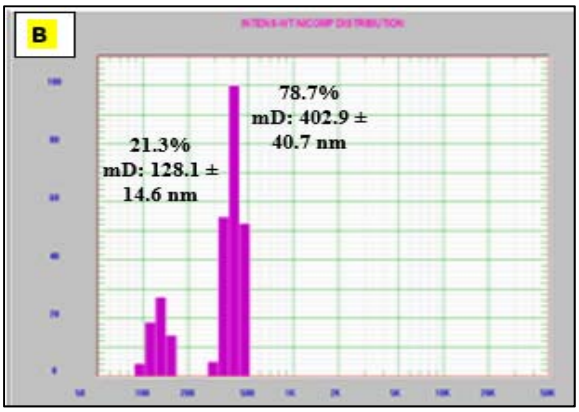


Fig 3B: DLS analysis: Particles size distribution in F2 (aq) of F0

In the extract F3 (Figure 3C), a different trend is observed, 3 types of particles (3 histograms) were identified by DLS characterizing 3 groups of particles in the solution with an overall size comprised between 0.2 and 4 µm.

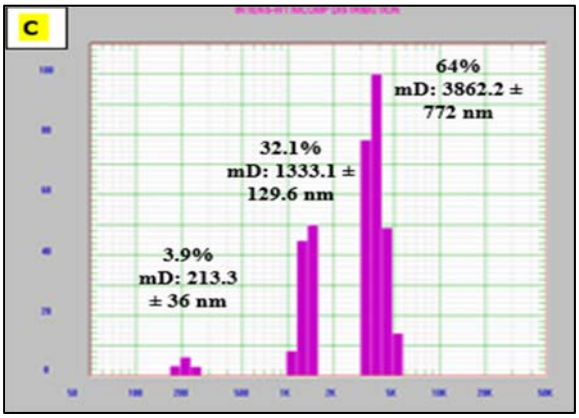


Fig 3 C: DLS analysis: Particles size distribution in F3 (aq) of F0

The average size distribution of the particles contained in F4 is between 0.1 and 0.4 µm as shown in Figure 3D and that is identical to those found in F2. Previous chemical analysis performed using TLC and DPPH antioxidant test on the same fractions (F1-F4) have shown in addition to their antioxidant potential, the presence of several PCD [13] with interesting pharmacological effects [5].

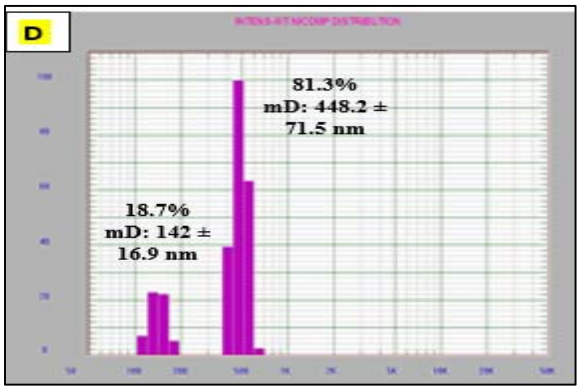


Fig 3D: DLS analysis: Particles size distribution in F4 (aq) of F0

DLS analyses have shown that the minimum and maximum size of particles in the suspension F0 based µemulsion (Labrasol-F0) are respectively ranged between 1 and 4 µm and are smaller than those found by [17] who obtained a diameter range of 1.4–40 µm in their µemulsion. The zone of µemulsification of Labrasol-F0 (O/W) in the pseudo-ternary phase diagram is also in accordance to the observations of [18]. Although, both microemulsions (µEm Labrasol-F0 and µEm T-F0) contain F0 as active extract and were stable for around 6 months (no phase separation was observed), it was useful to estimate the hygroscopic characteristics of all the extracts and µemulsions for their long term conservation using 97 % K_2SO_4 relative humidity [20]; the hygroscopic strength of F0-F4 and µemulsions in a saturated 97% K_2SO_4 RH was investigated for 259 h ~ 11 days and the results described by the following Figures 4A-C, Table 2 and Figure 5. Figure 4A shows that F0, F1, F2 and F4 progressively absorb water vapor in the surrounding environment from time 24 till 65 h and a constant mass of water is absorbed after 98 h where their saturation level is reached showed by a constant rate over time. Among the extracts, F3 seems to be the one absorbing more humidity, which is an indication that for a better formulation of this fraction for nasal administration for instant; the use of polymers or Propylene glycol will be required to increase viscosity [6] and its conservation should be under controlled conditions.

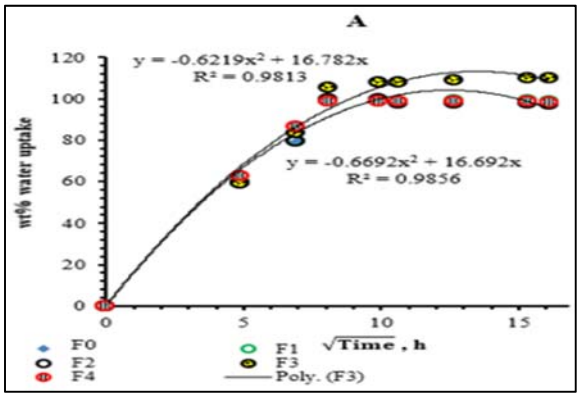


Fig 4A: Wt% water uptake vs Sqrt Time of exposition of F0-F4 to 97 % K_2SO_4 RH

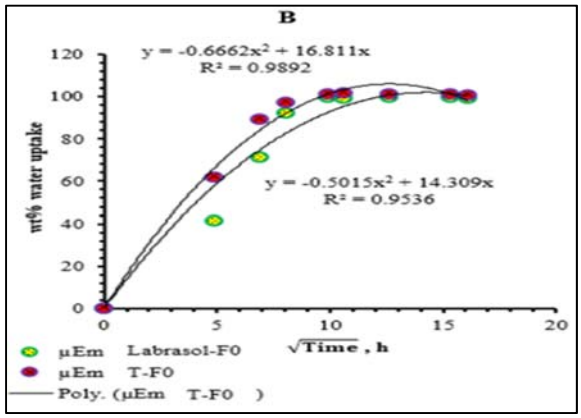


Fig 4B: Wt% water uptake vs Sqrt Time of exposition of µemulsions to 97 % K_2SO_4 RH

From Figure 4B, it is understandable that µemulsion Labrasol-F0 absorb less humidity from its environment than µemulsion T-F0 does. A constant mass of water uptake is noticed from time 65 h (after 3 days). A sample or formulation which easily uptake water vapor is more difficult to preserve and is expected to be the most instable over time, Figure 4C describes clearly that µemulsion Labrasol-F0 has the lowest hygroscopic strength while F3 and µemulsion T-F0 seem to have the highest.

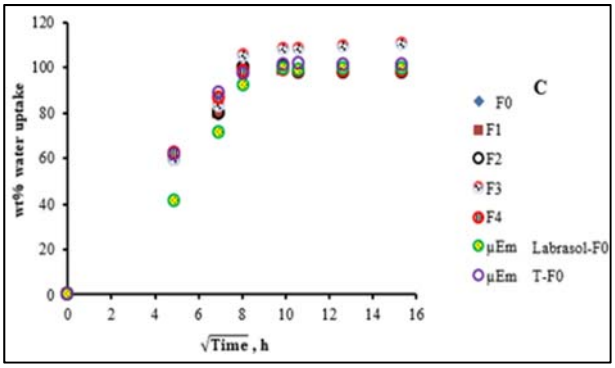


Fig 4C: Wt% water uptake vs Sqrt Time of exposition of samples to 97 % K_2SO_4 RH *µEm: Microemulsion*

F1-F4 are fractions of F0 and both µemulsions contain F0 as indicated, Figure 5 show the different ratio of the mass of water uptake compare to F0, the lowest mass of water uptake (blue dot) corresponding to F0/ µemulsion Labrasol-F0 while the highest mass uptake is F0/F3 (green dot). The progressive amount of water uptake by the samples before reaching a steady state may be related to the nature of their chemical components, this observation is in the same line [11] who link hydroscopic properties to chemical structure of compounds.

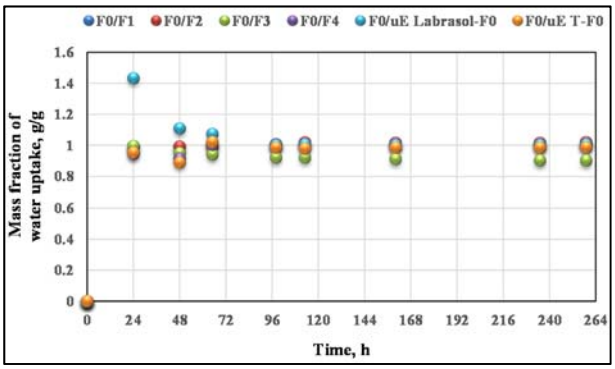


Fig 5: Compared mass fraction of water uptake over time in 97% K_2SO_4 RH

Even though, hygroscopic property of an extract, phytocompounds or formulation does not affect its pharmacological activity in the short term, it informs on the conservation measures to consider when dealing with such sample in order to preserve it biological activities. Table 2 compares different hygroscopic values in all the samples used.

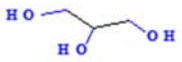
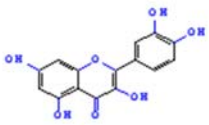
Table 2: Comparative hygroscopic values of extracts and formulations in 97 % K_2SO_4 RH

Relative Humidity In 97 % K_2SO_4							
F0	**F1**	**F2**	**F3**	**F4**	**µEmulsion (O/W) Labrasol-F0**	**µEmulsion (W/O) T-F0**	**Time, h**
0	0	0	0	0	0	0	0
59	59.6	59.6	59.2	62.4	41.2	61.9	24
79	79.6	79.6	83.2	86.4	71.2	88.9	48
99	98.8	100	105.2	98.5	92.2	97	65
99.6	98.9	99.8	108	98.9	99.6	101	98
99.5	98.8	97.6	107.9	98.5	99.1	101.6	113
99.4	99	97.8	108.9	98.5	99.3	100.9	160
99.4	99	97.8	110.1	98.6	99.3	100.9	235
99.4	98.6	97.4	110.1	98.4	98.9	100.7	259

Values: % water uptake by each sample over time

Microemulsions are constantly indexed to be one of the best drug vehicle for brain, nasal and skin drug delivery systems [21] because of their particular properties and composition, they are favorable to enhance drug diffusion and delivery across biological membranes (Table 3).

Table 3: Description of Labrasol-F0 µemulsion and safeness

Compounds name		Chemical structure, formula & molar mass	*LogPow	Plants Origin
Labrasol : (Surfactant) **And composition:**	**Glycerol** Humectant, polyol	 **Formula:** $C_3H_8O_3$ **Molar mass:** 92.09 g/mol	-1.8 (Hydrophilic)	Soybeans and palm
Enhance drug solubility, increase absorption and drug diffusion across biological membranes, harmless and environmentally friendly substances.	**Caprylic acid** Saturated fatty acid	 **Formula:** $C_8H_{16}O_2$ **Molar mass:** 144.21 g/mol	3.05 (Lipophilic)	In Coconut and palm kernel oil
	Oleic acid unsaturated fatty acid	 **Formula:** $C_{18}H_{34}O_2$ **Molar mass:** 282.47 g/mol	7.68 (Lipophilic, highly hydrophobic)	In olive and safflower oil
Quercetin (Polyphenol, Flavonoid, antioxidant, Safe)		 **Formula:** $C_{15}H_{10}O_7$ **Molar mass:** 302.236 g/mol	1.81 (Hardly water soluble)	In *Solanum aethiopicum* flower stalk (Kouasi et al., 2015)

*Values of LogPow (Octanol-water partition): Drugbank, 230516, www.drugbank.ca/drugs/

Color in chemical structures: Hydrophilic groups in blue and lipophilic groups in black

Further investigations will bring in more details about different applications on µemulsion Labrasol-F0, F0-F4 and their anti-inflammatory activities of will need to be assessed based on reported methods [22] in order to correlate scientific data and the use in traditional medicine of *S. aethiopicum* in Côte d'Ivoire. Microemulsion Labrasol-F0 was well formulated and totally dissolve as showed in Figure 2. It can be explain by the fact that the surfactant Labrasol contains both hydrophilic and hydrophobic substances (Table 3) as well as F0 and between hydrophilic groups, the interactions are favorable while hydrophobic interactions occur meanly between hydrocarbon chains and the aromatic rings of phytochemicals contain in F0 as observed by [23] who stated that interaction with Quercetin are dominated by hydrophobic interactions.

After 24 and 48 h treatment of Jurkat cells with 25 mg/ml of F0, the preliminary effect of F0 on these cancer cells, monitored by optical microscopy is describe by images in Figure 6. Jurkat cells generally used to study acute T cell leukemia are suspension cells; as observed in the control cells, they seem not stressed and proliferate normally from 24 to 48 h but some changes are observed in treated cells where it is noticed inhibition of cells growth and proliferation are by F0. The cancer cells undergo an agglomeration, the anti-proliferative and necrotic effect of F0 is more pronounced after 48 h of treatment.

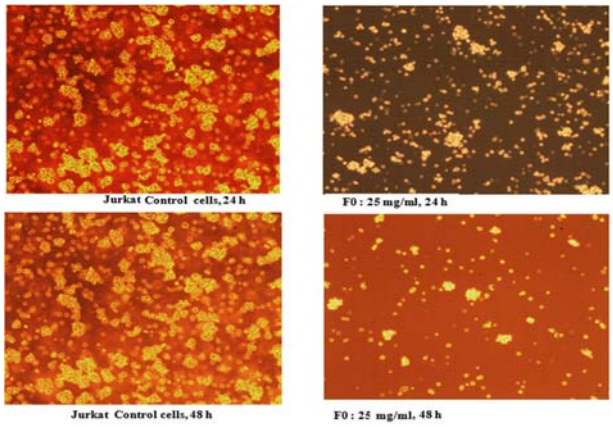


Fig 6: Antiproliferative effet de F0 on Leukemia Jurkat cells

The observed anticancer effects of F0 may be linked to its richness in several bioactive phytochemicals as reported [13, 7]. Further LC-MS, GC and MNR analyses for additional chemical characterization of F0-F4 will be very useful.

4. Conclusion

Two microemulsions were successfully formulated using F0 as mean active extract, the µemulsion Labrasol-F0 appears to be the most stable and promising; no coalescence and phase separation was observed over time. Fractions' DLS analysis has showed that F0 used in the formulation of the µemulsions has particles sizes ranged from 0.1 to 4 µm, however µemulsion T-F0 and F3 require more strict conditions for their better conservation. F0 induces growth inhibition and necrotic effects on Jurkat cells and can be further investigated in the treatment of on acute T cell leukemia.

5. Conflict of interest

The author(s) declare that this article has no conflicts of interest.

6. Acknowledgment

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