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Promising anticancer effects of Lycopene extracted from pink guava *Psidiumguajava L.*, in combination with Apigenin, and in comparison with Resveratrol

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Abstract:

Background*: To treat cancer, micronutrients obtained from certain fruits appear to offer an alternative to the frequently toxic chemotherapy methods. We therefore focused on Lycopene and Apigenin, the main components in pink guava Psidiumguajava L, and Resveratrol, a reference biomolecule with recognized anti-oxydant, neuroprotective and anticancer properties. Our aim was to assess and compare their antiproliferative action on LNCaP cells (prostate cancer) and UACC257 cells (melanoma).*

Materials and Methods*: Micronutrients were studied separately first and then in combination, using MTT assays. Confocal microscopy using anti-PCNA and anti-5-α-reductase primary antibodies enabled to evaluate the antitumoral pathway of Lycopen alone or in combination with Apigenin or Resveratrol, on prostate cancer cell.*

Results*: Lycopen alone has a selective impact on LNCaP, and Apigenin alone has a remarquableantiproliferative but non selective antiproliferative effect compared to others. Lycopene-Apigenin combination has a strong, more effective and non-toxic anti-prostate and anti-melanoma activity than the overall biomolecules used separately and in combination.*

Conlcusion*: Our study demonstrates the strong synergistic anti-cancer activity of Lycopene coupled with Apigenin, and the interest of the tropical pink guava named Psidiumguajava L in which these two micronutrients are naturally associated.*

Keywords: *Lycopene, Apigenin, Resveratrol, Prostate cancer, Psidiumguajava L, Melanoma.*

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I. INTRODUCTION

Melanoma has a high world-wide incidence.Exposure to UV rays is a predominant factor in the development of melanoma [2]. Prostate cancer is the second most widespread cancer in the world and is highly frequent in the Caribbean Basin, including the French West Indies, with a higher mortality rate than in mainland France [3]. This may primarily be due to genetic factors, but is also to the presence of an endocrine disruptor used in pesticides in banana agriculture – chlordecone[4]. Prostate cancer and melanoma require intensive chemotherapy, which is poorly tolerated by the body [5]. Numerous studies have indeed demonstrated the antioxidant and anti-proliferative effects of cytotoxic micronutrients, include carotenoids or phenolic compounds, extracted from fruits and vegetables biodiversity [6]–[8][9]–[11]. Specific combinations of phytochemicals are considered more effective than individual molecules in preventing and combating certain illnesses, including cancer. This shows the need to study the synergies between active plant compounds through experiments with plant extracts. Tropical fruits, and among them the pink guava *Psidiumguajava L.*, are a source of antioxidants such as polyphenols, carotenoids, and vitamins [12].Resveratrol (trans-3,5,4'-trihydroxystilbene), is a natural polyphenol found in a large variety of fruits such as grapes, berries and peanuts, and in some medicinal plants [13]. It is involved in numerous metabolic and cell signaling pathways, inducing apoptosis and resistance to oxidative stress and to chronic inflammation [13]. Itis widely used because of its

recognized antioxidant [14], neuroprotective[15], [16] and anti-cancer properties. Lycopene (ψ,ψ-Carotene) has been studied for some time now in tomatoes or watermelon. It is the major carotenoids found in *Psidiumguajava L.*[17], [18]. It is particularly effective for the treatment of degenerative illnesses and cardiovascular diseases [19]. The literature places Lycopene among the best antioxidants [20], [21]. Apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), also found in large quantities in *Psidiumguajava L.*, is a polyphenol of the flavonoid subclass which have beneficial effects for combating thyroid cancer [22].Its antiproliferative and antiapoptotic effects are well described [23]–[27].Interestingly, Apigenin and Lycopene have demonstrated benefits to prevent premature damage of aging of the skin or development of skin cancer [28]–[31], and also to figh against frequent cancers such as prostate cancer [32]–[36] and breast cancer [37]–[39]. All of these biomolecules have been studied, for the most part, separately but not as combinations. Combined, they might contribute new benefits as regards to effective treatment options. Apigenin and Lycopene are naturally combined in pink guava. This present study aims to examine separately and in combinations, the antiproliferative action of Lycopene, Apigenin, and Resveratrol on prostate (LNCaP) and melanoma (UACC257) tumor cell lines. Human Embryonic kidney cells (HEK293T) were used as control. We used a cell viability assay based on Optical Densitometry measurement of MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] oxydation, which is proportionate to cell metabolism and proliferation. We also used immunofluroescent confocal analysis on LNCaP cells,to evaluate pathways of anticancer and anti-androgenic properties of Lycopen alone and combined. On one hand, we detected the Proliferating Cell Nuclear Antigen (anti-PCNA) and on another hand, the 5-α-reductase enzyme.

II. MATERIAL AND METHODS

1. Plant materials

*Psidiumguajava L Cuba Enana*guava, one of the sweetest pink guava varieties, was obtained from the Martinique fruit orchard "Association Vergers et JardinsTropicaux (AVJT)". 175 pink guavas were harvested at the "turning" stage (transition from the mature to the ripe stage) and divided into five batches. The fruits were then cut into quarters, frozen in liquid nitrogen, and stored at -80°C until use. For extraction, the fruits were thawed the day before at +4°C, weighed, and ground in a Kenwood mill for 5 min at room temperature. The resulting puree was sieved (2mm) and aliquots were stored at -20°C. The preparation of guava puree in 100g portions is performed according to the technique described by [13].

2. Purification of Lycopen

Lycopene was extracted from pink guava and purified by HPLC (>95% purity), according to the method described by [12]. The peaks were identified by comparing their retention times with those of a commercially available tomato Lycopene standard (Sigma). Crystalline Apigenin 25mg and Resveratrol 100mg were obtained from Sigma.

3. Cell culture condition

Human embryonic kidney cells, HEK293T, were cultured in DMEM medium. Melanoma cell line UACC257 were obtained from NCI-60 and cultured in RPMI 1640. The LNCaP cell lines were obtained from IGR (Villejuif, France) and maintained in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum, 1mM pyruvate, 1% nonessential amino acids, 2mM glutamine, 100U/mL penicillin, and 100µg/mL streptomycin obtained from Invitrogen. Cells were maintained at 37°C under a humidified 5% CO_2 atmosphere.

4. Cell viability assay (MTT assay)

Cell line viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Sigma). 2500 HEK293T, LNCaP, and UACC257 cells were plated on a 96-well plate. They were incubated respecting cell culture conditionsfor 24, 48, and 72 hours with increasing ranges of concentrations of micronutrients separately or in combination. Concentration ranges have been chosen according to their physiological and usually used doses: Lycopene (0.5, 5 and 15µM), Apigenin (20, 40 and 80µM) and Resveratrol (10, 50 and 100µM). Each experiment was reproduced in triplet. At the end of the incubation period, cells were incubated with 0.5mg/mL of MTT for 1 or 2 hours and then resuspended in 100µL of DMSO. Viability was subsequently determined by measuring the absorbance at 450nm with a spectrophotometer (SpectraMax ABS, Molecular Devices).

5. Confocal immunofluorescence microscopy analysis applied on LNCaP

On one hand, anti-PCNA activity of chosen range concentration of Lycopen was dectected and compared to control HEK293T cells. On another hand, the anti-5-α-reductase activity of "Lycopen 15µM + Apigenin 80µM" and "Lycopen 15µM + Resveratrol 100µM" combinations were evaluated. Confocal analysis was performed as previously described using 1:200 dilution of adequate primary antibodies (Santa Cruz Biotechnology, CA) [42].

6. Statistical analysis

Data are presented as mean +/- standard error of the mean (SEM) of six independent experiments done in triplicate (n=6). The results were submitted to statistical comparison using two-way analysis of variance

(ANOVA), followed by Bonferroni multiple comparison test using Graph Pass Prism v.5. The differences were considered significant when $p<0.05$.

III. RESULTS

1. Effect of single molecules on viability

Lycopene being hydrophobic, it was resuspended in Tetrahydrofuran (THF) and then diluted in culture medium. This stage was not necessary for Apigenin and Resveratol. We checked that under our *in vitro* conditions, THF has no adverse effect on cell viability or proliferation. Therefore, all cell lines showed an expected characteristic of growth in the absence of treatment (data available not shown). Figure 1 reports data obtain for the MTT-assay on cell line treated by single molecules.Table 1 summarizes results of the treatments with a statistically significant effect compared to the control.

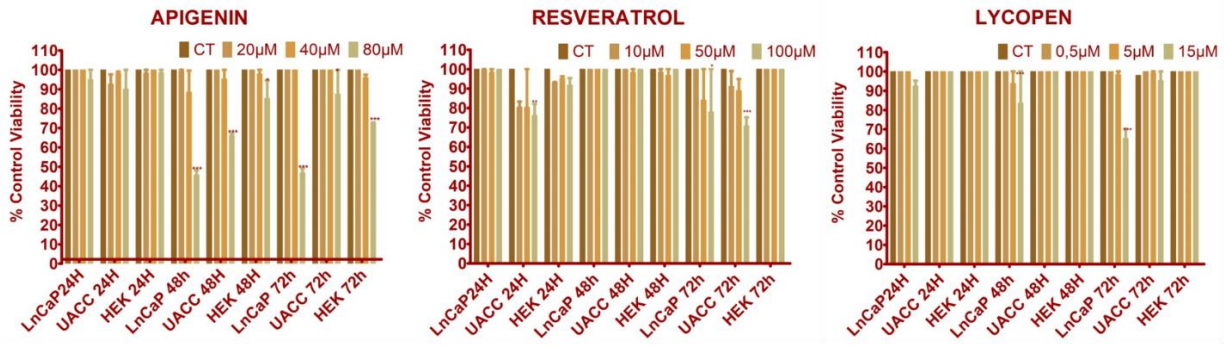


FIGURE 1. **Effect of treatments on cell lines viability** after 24, 48 and 72h of exposure with several concentration of molecules as assessed by MTT assays. Data are presented as mean ± standard error of the mean from 3 independents experiments. A significant difference between each concentration and the control evaluated using Bonferroni multiple comparison test. *p<0,05; **p<0,01; ***p<0,001.

	LnCaP			UACC257			HEK293T		
	concentration	*exposure time*	*relative decrease of viability*	*concentration*	*exposure time*	*relative decrease of viability*	*concentration*	*exposure time*	*relative decrease of viability*
APIGENIN	80µM	48h	55%	80µM	48h	35%	80µM	48h	15%
	80µM	72h	55%	80µM	72h	15%	80µM	72h	25%
LYCOPEN	15µM	48h	15%						
	15µM	72h	35%						
RESVERATROL	100µM	72h	20%	100µM	24h	25%			
				100µM	72h	30%			

Table 1.**Summary of statistically significant results of treatments** with different concentration of Apigenin, Lycopen and Resveratrol.

Only the maximum concentrations have a significant effect.The percentage of decrease in viability varies according to the exposure time, excepted for Apigenin exposure on UACC257 where the decrease observed at 72h of exposure were less important than at 48h. Apigenin treatment impacts all cell lines from 48h of exposure where a decrease of 55% in LnCaP, 35% in UACC257 and 15% in HEK is observed. After 72h, the rates reach respectively 55%, 15% and 25%. LnCaP is sensitive to treatment with all molecules from 48h of exposure. Treatment with Lycopen alone does not affect UACC257 and HEK293T but only LNCaP. Their percentage of viability decreased by 15% after 48h and 35% after 72h; It was only by 20% with the highest dose of Resveratrol after 72h of exposure.Resveratrol does not affect the HEK293T cell line. A diminution of 25% of the viability of UACC257 cells is observed after 24h of exposure, about 30% after 72h of exposure.

2. Comparison of the effects of combined molecules on viability

For the first time, we evaluated the synergic effect of Lycopene combinated with Apigenin or Resveratrol. To evaluate the optimal dosage of each molecule in the combination, we test several combinations of concentrations based on those previously chosen (Figure 2).Table 2 and 3 summarizes the results of the treatments with a statistically significant effect compared to control.

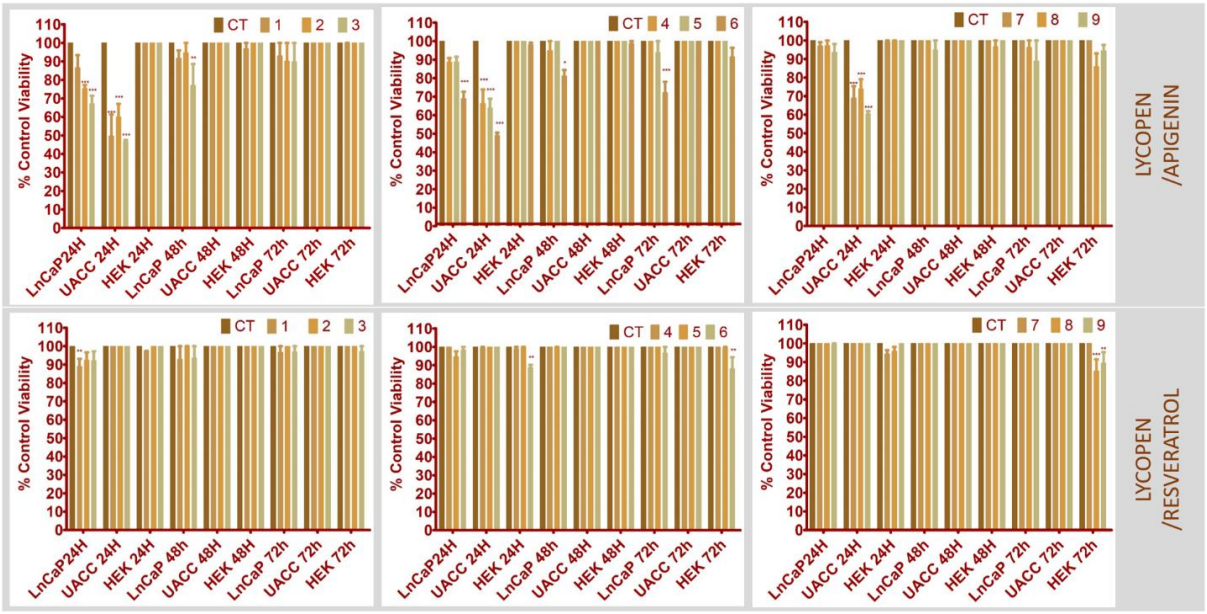


FIGURE 2. Effect of different combinations of molecule concentrations on cell lines viability after 24, 48 and 72h of exposure as assessed by MTT assays. Data are presented as mean ± standard error of the mean from 3 independents experiments. A significant difference between each concentration and the control evaluated using Bonferroni multiple comparison test. *p<0,05; **p<0,01; ***p<0,001.

	concentrations (µM)		**LnCaP**		**UACC257**		**HEK293T**	
	LYCOPEN	**APIGENIN**	*exposure time*	*relative decrease of viability*	*exposure time*	*relative decrease of viability*	*exposure time*	*relative decrease of viability*
1	15	20			24h	50%		
2	15	40	24h	25%	24h	40%		
3	15	80	24h	35%	24h	55%		
			48h	25%				
4	5	20			24h	35%		
5	5	40			24h	35%		
6	5	80	24h	30%	24h	50%		
			48h	20%				
			72h	30%				
7	0,5	20			24h	30%		
8	0,5	40			24h	25%		
9	0,5	80			24H	40%		

Table 2.Summary of statistically significant results of treatments with different concentration combinations of Apigenin and Lycopen.

	concentrations (µM)		**LnCaP**		**UACC257**		**HEK293T**	
	LYCOPEN	***RESVERATROL***	*exposure time*	*relative decrease of viability*	*exposure time*	*relative decrease of viability*	*exposure time*	*relative decrease of viability*
1	15	10	24h	10%				
2	15	50						
3	15	100						
4	5	10						

5	5	50						
6	5	100					24h	10%
							72h	10%
7	0,5	10						
8	0,5	50					72h	15%
9	0,5	100					72h	10%

Table 3.Summary of statistically significant results of treatments with different concentration combinations of Resveratrol and Lycopen.

When Apigenin and Lycopen are combined, no significant effect was observed on the HEK293T line, whereas treatment with the highest dose of Apigenin alone showed a decrease in their viability (15% from 48h and 25% after 72h).Whatever the combination of concentrations, UACC257 are significantly impacted by the treatments from 24h of exposure. While Lycopen alone had no significant effect on this line, the combination with Apigenin resulted in a decrease in viability ranging from 25% with combination n°8 to 55% for combination n°3. Combination n°3 corresponds to the combination of the highest concentrations of Apigenin (100µM) and Lycopen (15µM). Combination 8 combines the lowest concentration of Lycopen (0.5µM) with an intermediate concentration of Apigenin (40µM).

LnCaP cells viability is significantly affected by Combinations 2, 3 and 6. In combination 2, the highest concentration of Lycopen (15µM) combined with an intermediate concentration of Apigenin (40µM) resulted in a 25% decrease in cell viability after 24 hours of exposure, whereas it was 15% after 48 hours of exposure with Lycopen alone. The combination n°3, of higher concentrations, allows obtaining an earlier but less efficient effect than with micronutrients alone. Combination n°6, associating the highest concentration of Apigenin with an intermediate concentration of Lycopen, leads to a constant decrease in the viability (30% maximum) of the LnCaP cells from 24h of exposure. This is comparable to the activity of Lycopen alone after 72 hours of treatment.

While Resveratrol alone impacted the cell viability of the tumoral celllines, in combination with Lycopen it had no effect at all on UACC257; But, the highest concentration of Lycopen (15µM), combined with the lowest concentration of Resveratrol (10µM), resulted in a 10% decrease in the viability of LnCaP cells after 24 hours of exposure. On the other hand, while neither Lycopen nor Resveratrol alone had any effect on HEK293T, in combination they significantly impacted the viability of this cell line. Combinations 6, 8 and 9 impact their viability. The lowest concentration of Lycopen (0.5µM), associated with 50 or 100µM of Resveratrol, is sufficient to observe at least a 10% decrease in HEK293Tviability after 72h of exposure. The lowest 5µM of Lycopen combined with the highest dose of Resveratrol (100µM) impacts theirviability at 24h and 72h of exposure, decreasing it by 10%.

3. Anti-prostate cancer evaluation of single or combined molecules using anti-PCNA and anti-α-reductase confocal analysis

Confocal immunofluorescence was used to evaluate HEK293T versus LNCaP cell proliferation in the presence and absence of different Lycopene concentrations (0.5-5 and 15µM) after exposure of 72 hours, using an anti-PCNA antibody (nuclear labeling) (Figure 3).

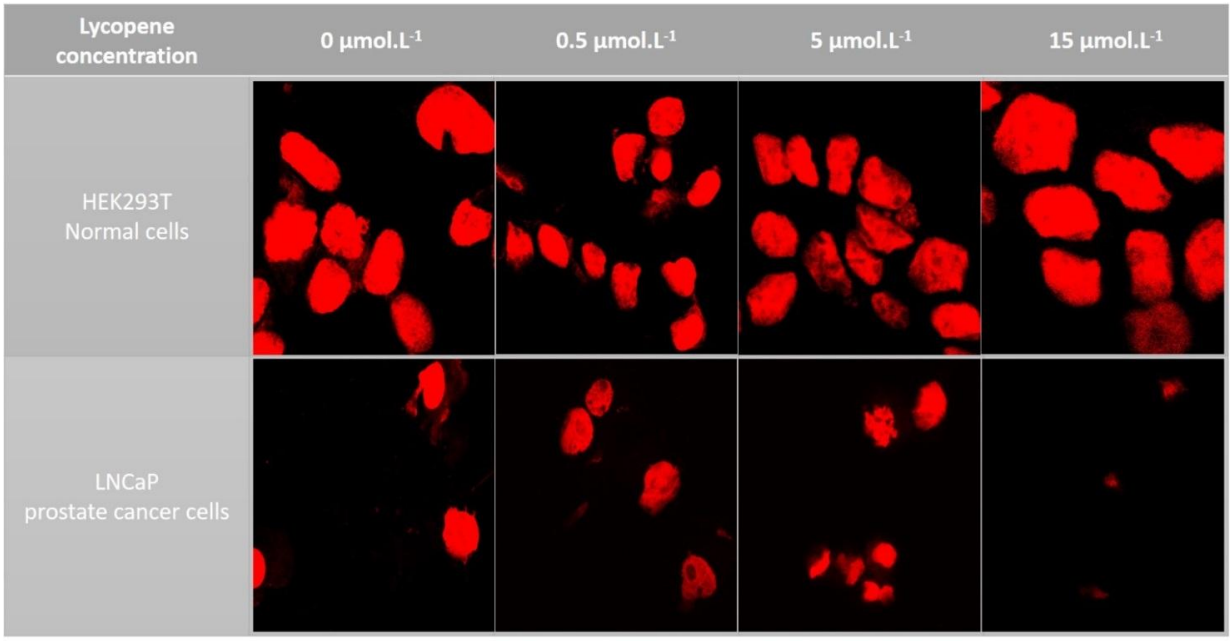


FIGURE 3. **Confocal evaluation of cell proliferation of LnCaP, using anti-PCNA antibody**. Normal HEK293T cells and prostate cancer cell LNCaP after 72 hours of exposure to increase Lycopene concentration range from 0.5-5-15µmol.L^{-1} versus non exposed cells.

Confocal detection of cytoplasmic 5-α-reductase enzyme was performed using anti-5-α-reductase antibodyafter 72h of exposition of LNCaP to combinations of 15µMLycopene and 80µMApigenin,or 100µMResveratrol compared to untreated cells (Figure 4).

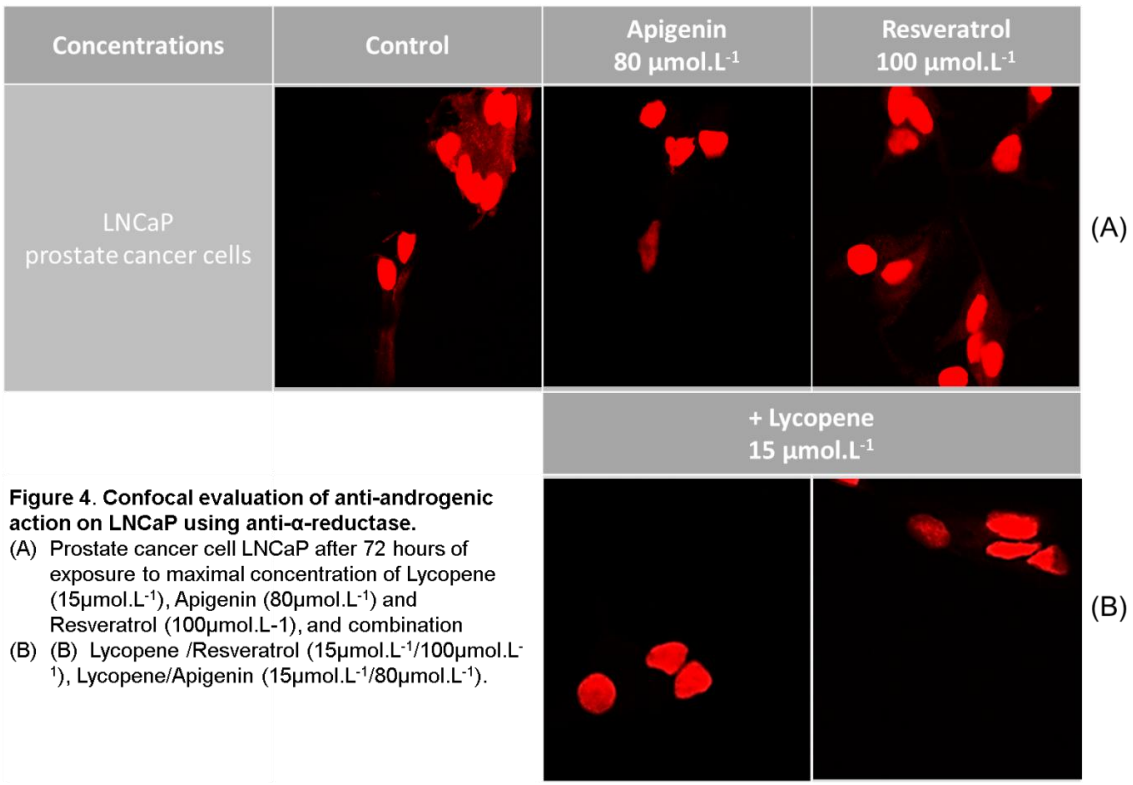


Figure 4. **Confocal evaluation of anti-androgenic action on LNCaP using anti-α-reductase.**
(A) Prostate cancer cell LNCaP after 72 hours of exposure to maximal concentration of Lycopene (15µmol.L^{-1}), Apigenin (80µmol.L^{-1}) and Resveratrol (100µmol.L-1), and combination
(B) (B) Lycopene /Resveratrol (15µmol.L^{-1}/100µmol.L^{-1}), Lycopene/Apigenin (15µmol.L^{-1}/80µmol.L^{-1}).

The results show that Lycopene has no effect on healthy HEK293T cells proliferation. Whereas a reduction in the proliferation of the prostate cancer cell LNCaP is detectable for a Lycopene concentration ranging from 0.5 to 15µMin comparison with untreated cells. The reduction in the PCNA signal increases with Lycopene amount until complete extinction of the signal obtained at 15µM Lycopene. This pigment therefore has no antiproliferative effect on healthy cells and acts specifically on prostate cancer cells.

When used separately, Lycopene and Apigenin exhibit inhibitory property for 5-α-reductaseand therefore, for 5-alpha-DHT which is responsible for the development of prostate cancer. The results confirmthe way of action ofthe combination of "Lycopene 15µM/Apigenin 80µM"."Lycopene 15µM/Resveratrol 100µM"combination partially inhibits the expression of the enzyme 5-α-reductase. Resveratrol alone has no effect on 5-α-reductaseandhas no more benefit when combined with Lycopene. The effect of this combination is essentially equivalent to the intrinsic activity of Lycopene alone.

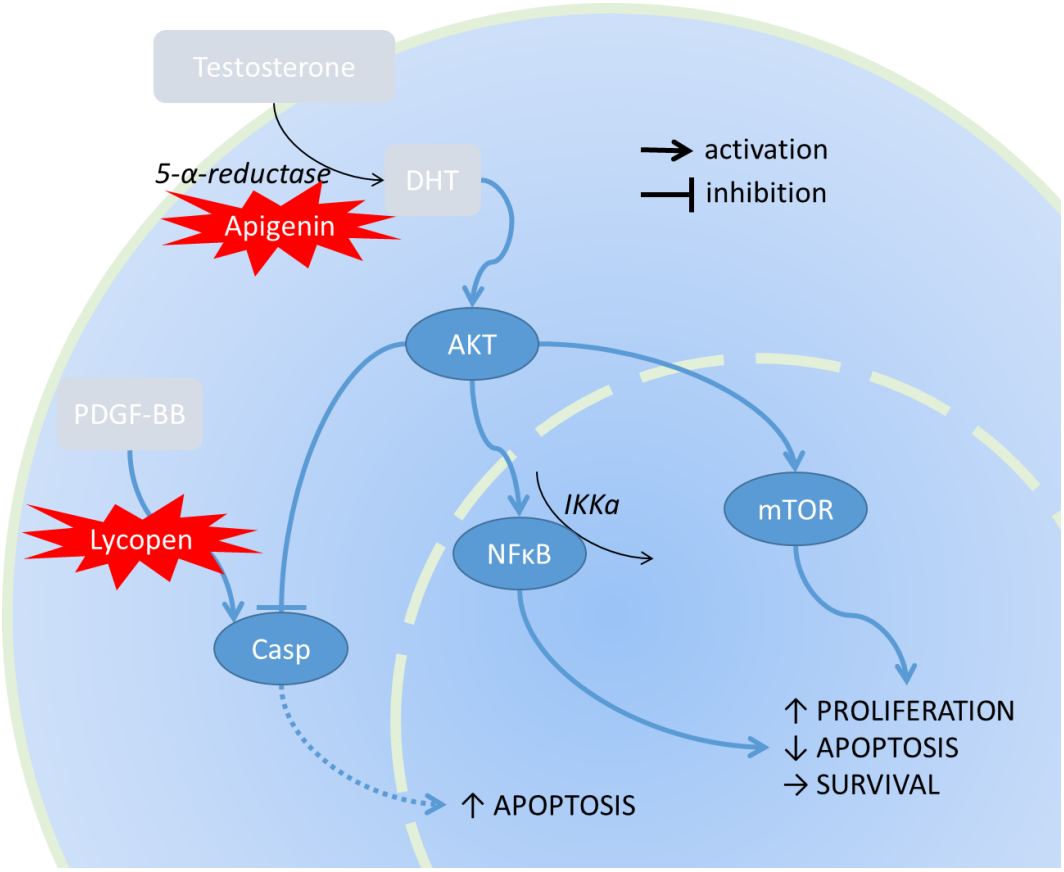
The "Lycopene/Apigenin" combination therefore appears to be the best possible combination for achieving total inhibition of the expression of 5-α-reductase. Furthermore, our results show a particularly targeted action of Apigenin on the expression of the enzyme.

IV. DISCUSSION

The aim of our study was to compare and evaluate the individual antitumoral effect of three different concentrations of Apigenin, Resveratrol andLycopene; but also for the first time the action of several combination of concentration of Lycopene with Apigenin or Resveratrol on prostate cancer cell lines and melanoma cell lines. This was performed using the MTT assay on both LNCaP and UACC257 cancer cell lines compared to a non-affected HEK293T control human kidney cell line. To evaluate signaling pathways involved in LNCaP decrease of viability, we realized confocal microscopy analysis with anti-PCNA and anti-5-α-reductase antibodies. The androgen 5-alpha-dihydrotestosterone, produced from testosterone through the action of the 5-α-reductase, is responsible for prostate cancer and benign prostatic hyperplasia [40]. PCNA is a protein which is involved in DNA replication and has cell cycle dependent properties. It has been widely used as a tumor marker for cancer cell progression and patient prognosis. The inhibition of PCNA can also result in suppression of cancer progression. Our study reveals several insights aboutthe inhibitory effect of alone or combined micronutrients on viability, proliferation, and 5-α-reductasepathway.

Firstly, the results of the single molecule treatment experiments show that after 72 hours of exposure, the highest concentrations tested are the most effective: 80µM Apigenin, 15µM Lycopen and 100µM Resveratrol.Of the 3 cell lines LnCPawere always impacted by treatments.Apigenin has the most marked antitumoral effectand anti-5-α-reductaseeffect on LNCaP, followed by Resveratrol.

Apigenin inhibits the cell cycle of keratinocytes by interrupting it at the G2/M stage [41] or by inhibiting apoptosis of keratinocytes exposed to UVB [31] or also by the inhibition of mTOR signaling involved in the development of skin cancer [42]. Its inhibition action on human prostatic tumor cell proliferation acts like an estrogen. It is considered partially mediated by ERB estrogen receptor [43]. In a similar way, blocking of IKKa kinase activity [23] or inactivation of Akt is thought to induce apoptosis of prostate cancer cells [24].Resveratrol is thought to inhibit the α-Melanocyte Stimulating Hormone signaling involved in melanoma invasiveness [16]. We were not able to detect an anti-androgenic effect of Resveratrol, whereas it is proven *in vivo* on transgenic mice developing adenocarcinoma of the prostate, or on transgenic adenocarcinoma mouse prostate, and also on prostate cancer xenograft models [17]–[19]. *In vitro* effects of this polyphenol on human prostate cancer cell lines, particularly LNCaP and PC-3M-MM2, was observed for a long incubation time, i.e. 96 hours [20]–[22]; What was not achieved in our experiments. Lycopene does not impacted Melanoma cell line viability. Lliterature reports indirect photoprotective effectsof Lycopen[40].Its inhibitory action on platelet-derived growth factor BB (PDGF-BB), possibly take place via the signaling pathways [41]. Lycopene is capable of interrupting the cell cycle by blocking cyclins such as D1, E and CDK4 [45].By the way, our experiments confirm results previously reported on anti-androgenic *in vitro*effect of Lycopen by inhibiting the expression of 5-α-reductase[44]–[46]. It have also been demonstrated in rat model [47]. Lycopene act as an antitumor agent by arresting cell proliferation and/or by inducing apoptosisreducing the risk of developing prostate tumor [42]–[44] [48] [53]. Schema 1 is a synthetic proposal of the molecular pathways of Lycopen compared to Apigenin.



Secondly, the results of the treatments with the associated molecules show that their addition does not mean the addition of their effects and also remind us that the combination of certain initially non-toxic molecules can be harmful. When combining Lycopene with Apigenin, an earlier antiproliferativeeffect is observed (from 24 hours of exposure) than when treated with molecules alone (at least 48h of exposure required). HEK293T were not impacted by long term exposure of this combination. A very high anti-androgenic activity is detected on prostate cancer cell line LNCaP. While no significant decrease in prostate cancer line viability is detected in the presence of 40µM Apigenin, the addition of 15µM Lycopen has an effect. This indicates that the efficacy of Apigenin is increased in the presence of Lycopen, and 40µM Apigenin is the minimum physiological concentration required in synergy with the higher dose of Lycopen to observe an effect *in vitro*. The greatest synergistic effect of this combination is detectable when the minimal 5µM concentration of Lycopen is added to the maximal 80µM concentration of Apigenin. At that combination of concentrations, the percentage of viability decreases almost to 30% for the prostate cancer cell line LNCaP, and 50% for melanoma cell line. But this effect does not persist significantly after 24h of exposure on UACC257.These observations reinforce the idea of a synergistic and non-toxic action between Lycopene and Apigeninthrought inhibition of the 5-α-reductase, and thereby the production of 5-alpha-DHT involved in prostatic hyperplasia. Resveratrol which initially had no effect on the controlcellsacquires some potential toxicity in the presence of low doses of Lycopen, but respecting certain dose combinations. The efficacy of Lycopen on the viability of LnCaP lines increases in the presence of the lowest dose of Resveratrol.

V. CONCLUSION

Our study confirms antiproliferative and antiprostatic selective activity of Lycopen. Apigenin appears to be an aggressive molecule regards to prostatic and skin cancer cells, and probably toxic in the long term exposure to the survival of healthy cells. When combined with Lycopen, its action seems to be more efficient and specifically directed against prostate and melanoma cancer cell lines and not against healthy ones, whereas the combination of Lycopen and Resveratrol can produce some toxicity towards them. Add an intermediate concentration of Apigenin to a low concentration of Lycopen is sufficient to affect*in vitro* prostate cancer cell survival through 5-α-reductase and PCNA inhibition.This work raises hopes and prospects for alternative therapies based on *Psidiumguajava* L pink guava, which contains these two micronutrients combined in a natural way.

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