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# Effect of a dietary supplement on the reduction of lymphedema-progression in mouse tail-cut model

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**Abstract. – OBJECTIVE: The aim of our study was to evaluate *in vivo*, in a mouse tail model of lymphedema, the effects of a dietary supplement, Garlive®, based on hydroxytyrosol from olive leaves, spermidine from rice seeds, hesperidin from citrus fruits and vitamin A. Hydroxytyrosol has anti-inflammatory, antioxidant and antimicrobial activities and inhibits leukotriene B4 generation; spermidine is able to inhibit the production of pro-inflammatory cytokines and mediators; hesperidin inhibits the secretion of pro-inflammatory cytokines: IFN-γ, IL-2, IL-4, IL-10; vitamin A deficiency was shown to induce inflammation and aggravate existing inflammatory states, whereas supplementation with vitamin A could ameliorate inflammation.**

**MATERIALS AND METHODS: The active compounds were included in tablets: 250 mg of olive leaf extract titrated in 10% hydroxytyrosol, 200 mg of citrus fruits extract titrated in 60% hesperidin, 10 mg of rice (*Oryza sativa*) seeds extract titrated in 1% spermidine and 0.8 mg of vitamin A. Mice of an inbred group were randomly selected and divided in the control group and drug-treated group. The wound necessary for lymphedema generation was made on the tail of each mouse 1 cm below the base of the trunk.**

**RESULTS: After surgical intervention, there was a gradual increase in the circumference of both ends of the wound. The control group showed higher increase of tail volume than the drug-treated group. The differences in tail swelling between the control group and the drug-treated group were significantly different. The peak of swelling was anticipated to the 6th day in the drug-treated group, whereas in the control group the peak was reached later on.**

**CONCLUSIONS: The tested drug prevented the induction of swelling from day 5th of wound creation and decreased the duration of swelling, favoring the wound healing.**

*Key Words:*
Lymphedema, Hydroxytyrosol, Spermidine, Vitamin A, Hesperidin.

## Introduction

Lymphedema is the abnormal accumulation of interstitial fluid due to inefficient fluid uptake and reduced lymphatic flow. It affects as many as 140 to 250 million people worldwide. Malformations in the lymphatic vasculature, injury, surgery, trauma or toxic damage may lead to swelling, mostly affecting the limbs[1]. Primary lymphedema is congenital, is caused by specific genetic mutations[2,3] and has a prevalence of ~1/100000[4]; whereas, secondary lymphedema is acquired (e.g., triggered by infections or surgery)[5]. Lymphedema can be regarded as a chronic inflammatory disease, in fact, accumulation of interstitial fluid induces infiltration of immune cells, activation of the inflammatory cascade, tissue fibrosis and build-up of adipose tissue[6,7]. This condition leads to increased risk of infections, debilitating pain, restricted movements and decrease of overall quality of life[8].

Leukotrienes are arachidonic acid derivatives produced by pro-inflammatory immune cells



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through the 5-lipoxygenase enzyme and implicated in the pathogenesis of lymphedema, producing strong inflammatory responses[9,10].

The aim of our study was to evaluate *in vivo*, in a mouse tail model of lymphedema[11], the effects of a dietary supplement, Garlive®, based on hydroxytyrosol (HT) from olive leaves, spermidine from rice seeds, hesperidin from citrus fruits and vitamin A. HT is a biophenol extracted from olives with anti-inflammatory, antioxidant and antimicrobial activities[12] that has the ability to inhibit the 5-lipoxygenase enzyme activity in leukocytes, blocking leukotriene B4 generation[13], thereby providing a potential safe option for preventing and treating inflammation[7,14]. Spermidine is able to inhibit the production of pro-inflammatory cytokines and mediators such as tumor necrosis factor-α, interleukin-1β, nitric oxide and prostaglandin E2[15]. The anti-inflammatory properties of hesperidin were already seen to be possibly effective at ameliorating lymphedema phenotype[16,17].

Vitamin A was found to be associated with lower levels of inflammation[18]. Interestingly, the physiologically active metabolite of vitamin A, the retinoic acid, is able to interact with the retinoic acid receptor-related orphan receptor C encoded by the gene *RORC*[19], that we have found to carry deleterious variants in lymphedema patients[20].

## Materials and Methods

### Tablets Composition

Each tablet contains active compounds: 250 mg of olive leaf extract titrated in 10% HT, 10 mg of rice (*Oryza sativa*) seeds extract titrated in 1% spermidine; 200 mg of bioflavonoids from citrus fruits titrated in 60% hesperidin, 0.8 mg of vitamin A.

### Animal Selection

Animals of inbred group were randomly selected and divided in 2 groups. The animals were in the range of body weight of 20-25 g. These 2 groups were named as Control Group (Group I) and Drug-Treated group (Group II). The study was approved by the Animal Ethics Committe of the Banara Hindu University (approval number Dean/2021/IAEC/2564).

### Surgical Intervention

The wound was created on the tail of each mice, 1 cm, below the base of the trunk. For this, a mark was made at 1 cm and 2 cm, from the body base (origin of tail). Between these marks, the skin was removed carefully to avoid any bleeding and to keep the tail cord intact. The cuts were made in aseptic-sterile conditions in the fuming hood. Before the surgical intervention, the animals were anaesthetized by intra-peritoneal ketamine injection. When the animals were in complete anaesthetic state, the animals were tied on the operation table, and tail was properly cleaned with ethanol (Figures 1 and 2). After surgery, Tincher-benzine was applied on the wound on 1st day. Each mouse was kept in separate cages with water and food *ad libitum*. We had full attention to avoid any necrosis, for 20 days of experiment.

### Drug Preparation

The 15 tablets were crushed and triturated in 5 ml of tween-20, and diluted to 50 ml with distilled water to get final ratio of 10% tween-20 in distilled water. The suspension was filtered on Whatman-1 filter paper and the final collected volume was 40 ml.

### Treatment Protocol

As per our experimental protocol, 1 tablet per mice per day was administered. We consid-

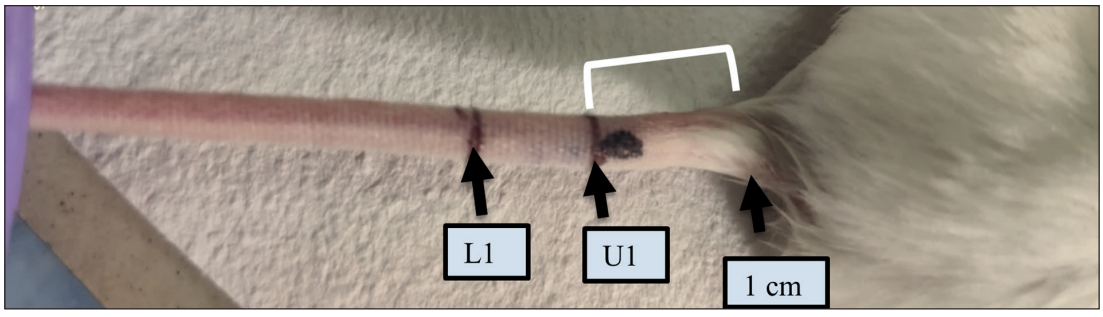


**Figure 1.** The area marked for the skin removal from the tail (1 cm away from the base of the trunk.

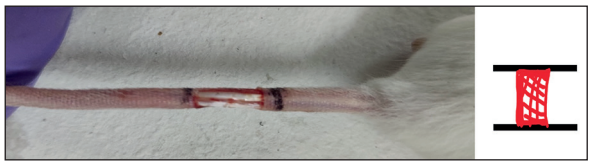

**Figure 2.** Circumferential annulus of skin between the 2 marks (marked in fig-1), 1 cm distal to the tail base, was removed from the tail. On the ventral side of the tail, dermal flap 4-mm$^2$ was excluded from removal, to maintain the tail health. (Note: if 100% skin is removed then there was significant necrosis in tail).

ered 30 g as the average body weight, and the volume of 2.67 ml of the drug-filtrate was calculated, accordingly, for oral administration. This volume was high for a single dose, causing stomach distension, therefore the volume was split in 2 equally-subdivided doses. One dose was given at 10 A.M., the other dose was given at 5 P.M.

### *Measurements*

After 24 hours, the thickness of tail on each end of the wound was measured by digital Vernier calliper. This data was considered as the diameter of the swelled tail and the circumference of that end of the wound was calculated by using the formula $2\pi R$. The photograph of each day was also recorded. For calculation of volume of wound swelling, the formula for determination of volume of a cone was applied:

$$V = \frac{h\,(C_1^2 + C_1C_2 + C_2^2)}{12\pi}$$

Where, $C_1$ is circumference of proximal end and $C_2$ was the circumference of distal end. "h" is the height, which is 1 cm here, as the wound ends were 1 cm apart (Figure 1).

**Figure 3.** A 4-mm$^2$ dermal flap located at the ventral side was excised from the tail by use of a surgical blade.

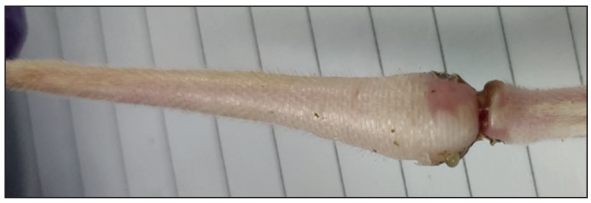

**Figure 4.** Ventral side of the tail showing highest thickness on the day 10$^{th}$ in the experimental control group.

## Results

After surgical intervention there was a gradual increase in the circumference of both end of the wound. The proximal end showed more swelling than the distal end of the wound (Figures 1-6). When we calculated the volume of the wound a significant increase in volume was recorded. All the increments were time-dependent. The control group showed higher increase of tail volume than the drug-treated (DT) group and the highest swelling was recorded on 10$^{th}$-11$^{th}$ day and was maintained up to the 15$^{th}$ day. Thereafter, there was a slight gradual decrease in swelling as recorded up to the 20$^{th}$ days (Tables I-III; Figures 7-9). In the drug-treated mice, the peak of swelling was found between 6$^{th}$-7$^{th}$ day with rapid decrease up to 20 days. The differences in tail swelling between control and DT groups were significantly different. A higher swelling degree in the first 5 days of experimentation in the DT than in the control was observed, although this increment was not significant. Thereafter, on 6$^{th}$ day onward, there was a rapid significant reduction of swelling, as indicated by gradual increase in the "t" value. Finally, the peak of swelling was anticipated to the 6$^{th}$ day in the DT group, whereas in the control group the peak was on the 10$^{th}$-11$^{th}$ day (Tables I-III). The differences in swelling among treated and untreated mice could also be seen at the histological examination with hematoxylin/eosin staining of tails (Figure 10). In the untreated mice, apparently, there was a marked inflammatory response, leukocyte infiltration and increased collagen deposition compared to the treated mouse.

## Discussion

We included HT, spermidine, hesperidin and vitamin A for their role in the modulation of pathways involved in the pathophysiology of lymphedema[7].

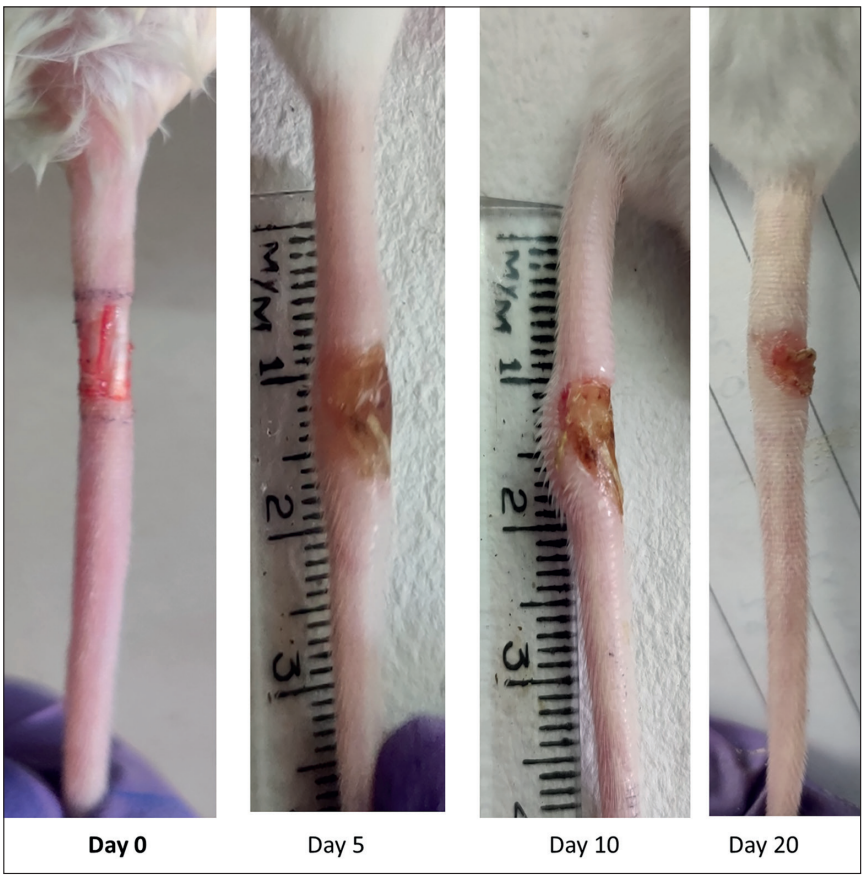


**Figure 5.** Changes in swelling of mouse tail at different days in drug treated group (n=10).

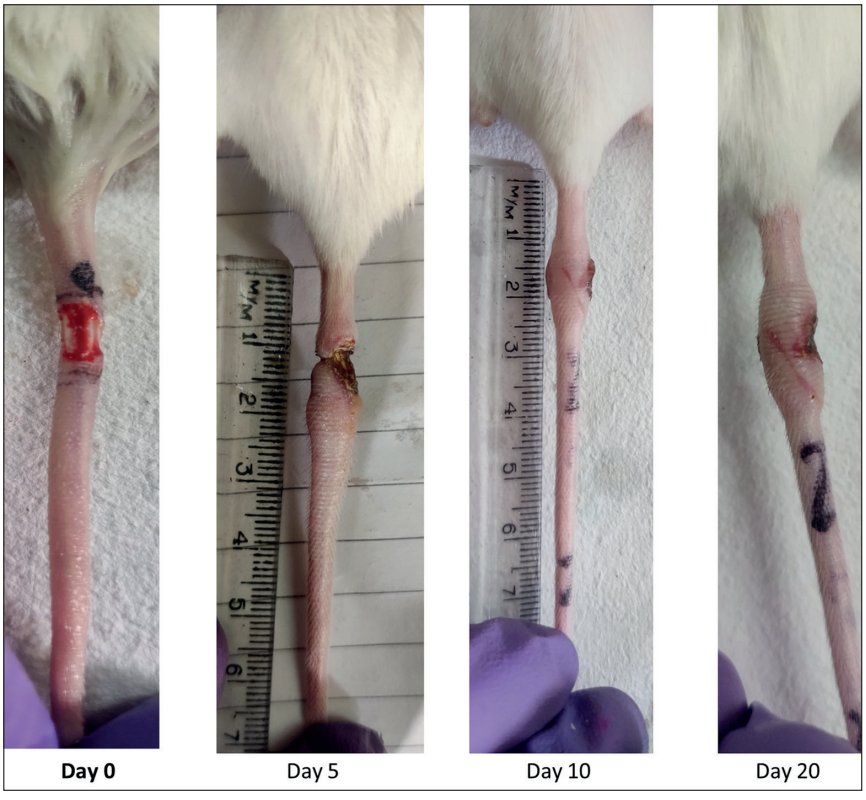


**Figure 6.** Changes in swelling of mouse tail at different days in the control group (n=10).

**Table I.** Kinetics of circumference of swelling of the proximal end of the wound of the control group vs the drug-treated group.

| Days | Control (n = 10) | | | Drug treatment (n = 10) | | | *t*-Test value: paired two sample for means. (control *vs.* drug-treated group) | | Change (cm) | % change in lower end |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (cm) | SD | SE | Mean (cm) | SD | SE | P (T<=*t*) one-tail | P (T<=*t*) two-tail | | |
| 1 | 1.247 | 0.083 | 0.026 | 1.231 | 0.063 | 0.020 | 0.453 | 0.906 | 0.015 | 1.22% |
| 2 | 1.348 | 0.100 | 0.032 | 1.430 | 0.052 | 0.016 | 0.235 | 0.470 | -0.082 | -6.08% |
| 3 | 1.464 | 0.110 | 0.035 | 1.543 | 0.036 | 0.012 | 0.261 | 0.521 | -0.079 | -5.39% |
| 4 | 1.516 | 0.107 | 0.034 | 1.638 | 0.049 | 0.015 | 0.184 | 0.368 | -0.122 | -8.07% |
| 5 | 1.573 | 0.110 | 0.035 | 1.653 | 0.083 | 0.026 | 0.312 | 0.623 | -0.080 | -5.06% |
| 6 | 1.837 | 0.106 | 0.034 | 1.555 | 0.092 | 0.029 | 0.052 | 0.104 | 0.283 | 15.38% |
| 7 | 1.743 | 0.117 | 0.037 | 1.509 | 0.109 | 0.034 | 0.082 | 0.164 | 0.234 | 13.45% |
| 8 | 1.876 | 0.115 | 0.036 | 1.450 | 0.121 | 0.038 | 0.012 | 0.025 | 0.426 | 22.73% |
| 9 | 2.005 | 0.112 | 0.035 | 1.126 | 0.193 | 0.061 | 0.001 | 0.002 | 0.879 | 43.83% |
| 10 | 2.104 | 0.112 | 0.036 | 1.050 | 0.213 | 0.067 | 0.001 | 0.001 | 1.054 | 50.08% |
| 11 | 2.203 | 0.074 | 0.023 | 1.021 | 0.219 | 0.069 | 0.001 | 0.001 | 1.182 | 53.65% |
| 12 | 2.135 | 0.070 | 0.022 | 1.005 | 0.221 | 0.070 | 0.001 | 0.002 | 1.130 | 52.93% |
| 13 | 2.126 | 0.070 | 0.022 | 0.997 | 0.218 | 0.069 | 0.001 | 0.002 | 1.129 | 53.09% |
| 14 | 2.098 | 0.061 | 0.019 | 0.989 | 0.217 | 0.069 | 0.001 | 0.002 | 1.109 | 52.84% |
| 15 | 2.018 | 0.068 | 0.022 | 0.968 | 0.217 | 0.068 | 0.001 | 0.002 | 1.050 | 52.02% |
| 16 | 1.956 | 0.075 | 0.024 | 0.945 | 0.211 | 0.067 | 0.001 | 0.002 | 1.011 | 51.69% |
| 17 | 1.864 | 0.075 | 0.024 | 0.915 | 0.204 | 0.065 | 0.001 | 0.002 | 0.949 | 50.90% |
| 18 | 1.804 | 0.091 | 0.029 | 0.890 | 0.199 | 0.063 | 0.001 | 0.001 | 0.914 | 50.66% |
| 19 | 1.753 | 0.089 | 0.028 | 0.874 | 0.196 | 0.062 | 0.001 | 0.001 | 0.879 | 50.13% |
| 20 | 1.676 | 0.082 | 0.026 | 0.855 | 0.197 | 0.062 | 0.001 | 0.002 | 0.821 | 49.00% |

SD = standard deviation; SE = standard error.

**Table II.** Kinetics of circumference of swelling of the proximal end of the wound of the control group vs the drug-treated group.

| Days | Control (n = 10) | | | Drug treatment (n = 10) | | | *t*-Test value: paired two sample for means. (control *vs.* drug-treated group) | | Change (cm) | % change in lower end |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (cm) | SD | SE | Mean (cm) | SD | SE | P (T<=*t*) one-tail | P (T<=*t*) two-tail | | |
| 1 | 0.389 | 0.015 | 0.005 | 0.374 | 0.016 | 0.005 | 0.259 | 0.518 | 0.015 | 3.95% |
| 2 | 0.408 | 0.017 | 0.005 | 0.422 | 0.010 | 0.003 | 0.268 | 0.537 | -0.015 | -3.61% |
| 3 | 0.421 | 0.017 | 0.005 | 0.435 | 0.009 | 0.003 | 0.247 | 0.494 | -0.015 | -3.50% |
| 4 | 0.434 | 0.017 | 0.005 | 0.444 | 0.014 | 0.004 | 0.331 | 0.663 | -0.010 | -2.30% |
| 5 | 0.439 | 0.017 | 0.005 | 0.436 | 0.018 | 0.006 | 0.446 | 0.893 | 0.003 | 0.73% |
| 6 | 0.449 | 0.016 | 0.005 | 0.443 | 0.020 | 0.006 | 0.401 | 0.803 | 0.006 | 1.43% |
| 7 | 0.464 | 0.017 | 0.005 | 0.447 | 0.026 | 0.008 | 0.302 | 0.605 | 0.017 | 3.64% |
| 8 | 0.475 | 0.018 | 0.006 | 0.431 | 0.031 | 0.010 | 0.134 | 0.268 | 0.044 | 9.36% |
| 9 | 0.492 | 0.019 | 0.006 | 0.360 | 0.041 | 0.013 | 0.012 | 0.024 | 0.132 | 26.84% |
| 10 | 0.502 | 0.021 | 0.007 | 0.334 | 0.047 | 0.015 | 0.006 | 0.012 | 0.168 | 33.46% |
| 11 | 0.507 | 0.021 | 0.007 | 0.328 | 0.048 | 0.015 | 0.005 | 0.010 | 0.180 | 35.44% |
| 12 | 0.504 | 0.021 | 0.007 | 0.318 | 0.047 | 0.015 | 0.004 | 0.008 | 0.187 | 37.01% |
| 13 | 0.513 | 0.013 | 0.004 | 0.316 | 0.046 | 0.015 | 0.003 | 0.007 | 0.197 | 38.41% |
| 14 | 0.511 | 0.012 | 0.004 | 0.313 | 0.046 | 0.014 | 0.003 | 0.005 | 0.198 | 38.73% |
| 15 | 0.498 | 0.015 | 0.005 | 0.305 | 0.046 | 0.015 | 0.004 | 0.008 | 0.193 | 38.75% |
| 16 | 0.490 | 0.014 | 0.004 | 0.296 | 0.042 | 0.013 | 0.002 | 0.004 | 0.194 | 39.66% |
| 17 | 0.480 | 0.015 | 0.005 | 0.286 | 0.040 | 0.013 | 0.002 | 0.003 | 0.194 | 40.46% |
| 18 | 0.472 | 0.017 | 0.005 | 0.284 | 0.038 | 0.012 | 0.001 | 0.003 | 0.188 | 39.86% |
| 19 | 0.464 | 0.016 | 0.005 | 0.279 | 0.037 | 0.012 | 0.001 | 0.002 | 0.185 | 39.95% |
| 20 | 0.459 | 0.015 | 0.005 | 0.276 | 0.038 | 0.012 | 0.001 | 0.002 | 0.183 | 39.78% |

SD = standard deviation; SE = standard error.

**Table III.** Kinetics of swelling of circumference of distal end of the wound of the control group vs the drug-treated group.

| Days | Control (n = 10) | | | Drug treatment (n = 10) | | | *t*-Test value: paired two sample for means. (control *vs.* drug-treated group) | | Change (cm) | % change in lower end |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (cm) | SD | SE | Mean (cm) | SD | SE | P (T<=*t*) one-tail | P (T<=*t*) two-tail | | |
| 1 | 0.389 | 0.015 | 0.005 | 0.374 | 0.016 | 0.005 | 0.259 | 0.518 | 0.015 | 3.95% |
| 2 | 0.408 | 0.017 | 0.005 | 0.422 | 0.010 | 0.003 | 0.268 | 0.537 | -0.015 | -3.61% |
| 3 | 0.421 | 0.017 | 0.005 | 0.435 | 0.009 | 0.003 | 0.247 | 0.494 | -0.015 | -3.50% |
| 4 | 0.434 | 0.017 | 0.005 | 0.444 | 0.014 | 0.004 | 0.331 | 0.663 | -0.010 | -2.30% |
| 5 | 0.439 | 0.017 | 0.005 | 0.436 | 0.018 | 0.006 | 0.446 | 0.893 | 0.003 | 0.73% |
| 6 | 0.449 | 0.016 | 0.005 | 0.443 | 0.020 | 0.006 | 0.401 | 0.803 | 0.006 | 1.43% |
| 7 | 0.464 | 0.017 | 0.005 | 0.447 | 0.026 | 0.008 | 0.302 | 0.605 | 0.017 | 3.64% |
| 8 | 0.475 | 0.018 | 0.006 | 0.431 | 0.031 | 0.010 | 0.134 | 0.268 | 0.044 | 9.36% |
| 9 | 0.492 | 0.019 | 0.006 | 0.360 | 0.041 | 0.013 | 0.012 | 0.024 | 0.132 | 26.84% |
| 10 | 0.502 | 0.021 | 0.007 | 0.334 | 0.047 | 0.015 | 0.006 | 0.012 | 0.168 | 33.46% |
| 11 | 0.507 | 0.021 | 0.007 | 0.328 | 0.048 | 0.015 | 0.005 | 0.010 | 0.180 | 35.44% |
| 12 | 0.504 | 0.021 | 0.007 | 0.318 | 0.047 | 0.015 | 0.004 | 0.008 | 0.187 | 37.01% |
| 13 | 0.513 | 0.013 | 0.004 | 0.316 | 0.046 | 0.015 | 0.003 | 0.007 | 0.197 | 38.41% |
| 14 | 0.511 | 0.012 | 0.004 | 0.313 | 0.046 | 0.014 | 0.003 | 0.005 | 0.198 | 38.73% |
| 15 | 0.498 | 0.015 | 0.005 | 0.305 | 0.046 | 0.015 | 0.004 | 0.008 | 0.193 | 38.75% |
| 16 | 0.490 | 0.014 | 0.004 | 0.296 | 0.042 | 0.013 | 0.002 | 0.004 | 0.194 | 39.66% |
| 17 | 0.480 | 0.015 | 0.005 | 0.286 | 0.040 | 0.013 | 0.002 | 0.003 | 0.194 | 40.46% |
| 18 | 0.472 | 0.017 | 0.005 | 0.284 | 0.038 | 0.012 | 0.001 | 0.003 | 0.188 | 39.86% |
| 19 | 0.464 | 0.016 | 0.005 | 0.279 | 0.037 | 0.012 | 0.001 | 0.002 | 0.185 | 39.95% |
| 20 | 0.459 | 0.015 | 0.005 | 0.276 | 0.038 | 0.012 | 0.001 | 0.002 | 0.183 | 39.78% |

SD = standard deviation; SE = standard error.

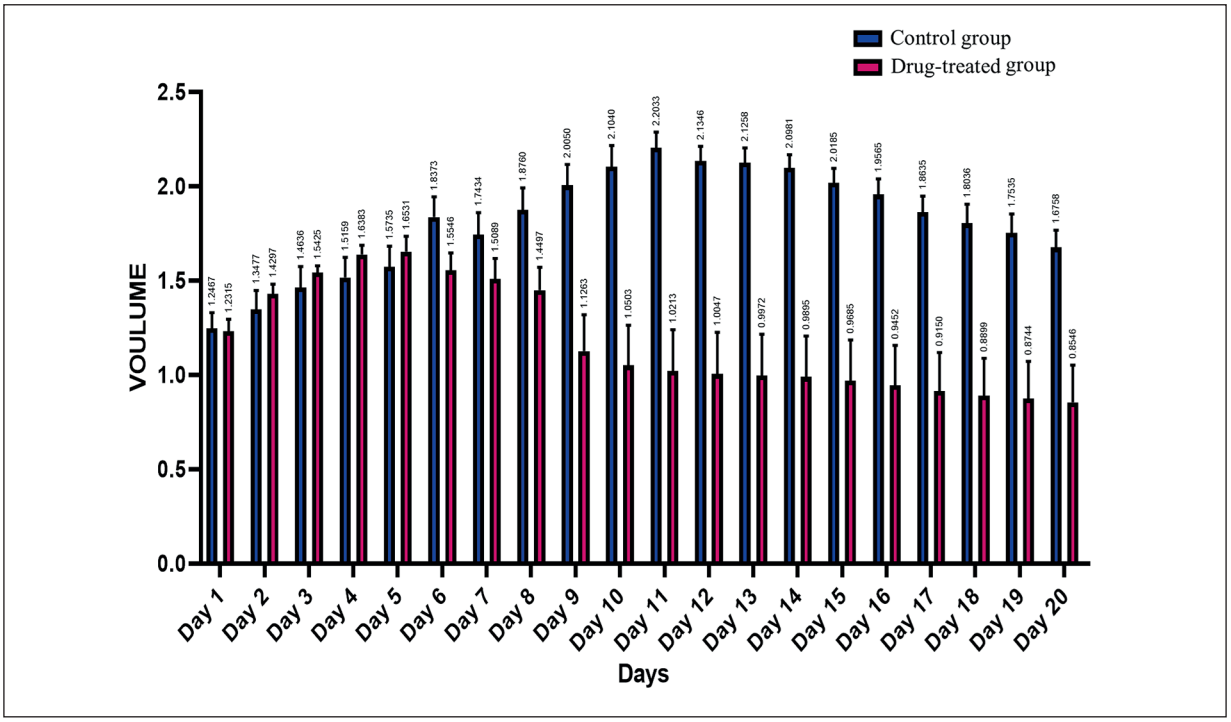


**Figure 7.** Graphical representation of change in volume of mice tails [Drug-treated group (*in red*) *vs.* Control group (*in blue*)]. See Table I for the statistical analysis.

HT inhibits the leukotriene B4 biosynthesis[21]. Leukotriene B4 is regarded as one of the most important molecule in the pathophysiology of lymphedema and its antagonism could ameliorate lymphedema phenotype[6,22]. Hesperidin has anti-inflammatory and immunomodulatory properties, as demonstrated in mice studies, and it was tested in patients with secondary lymphedema,

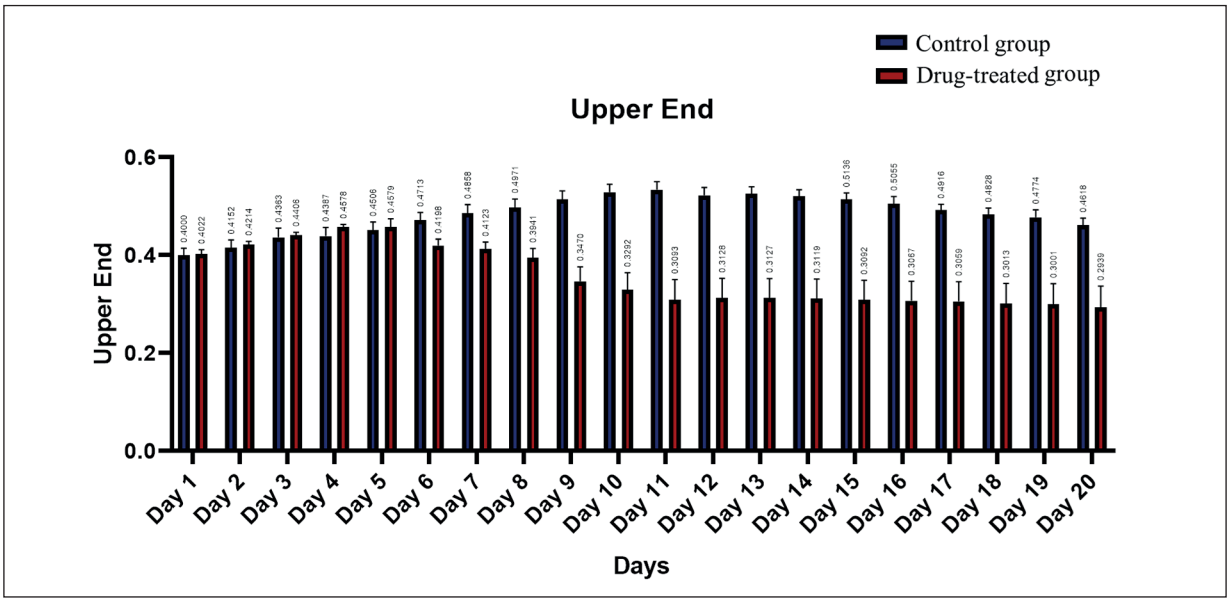


**Figure 8.** Graphical representation of changes in volume of the upper (*proximal*) end of mice tails [Drug-treated group (*in red*) *vs.* Control group (*in blue*)]. See Table II for the statistical analysis.

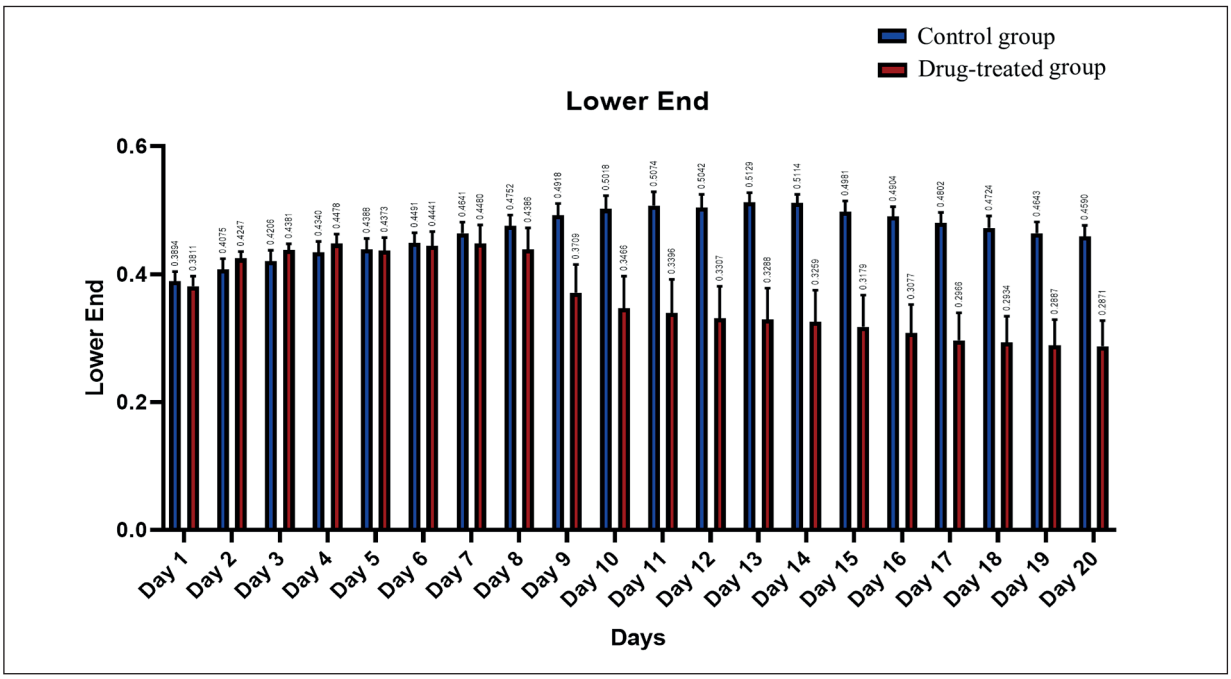


**Figure 9.** Graphical representation of changes in volume of the lower (*distal*) end of mice tails [Drug-treated group (*in red*) *vs.* Control group (*in blue*)]. See Table III for the statistical analysis.

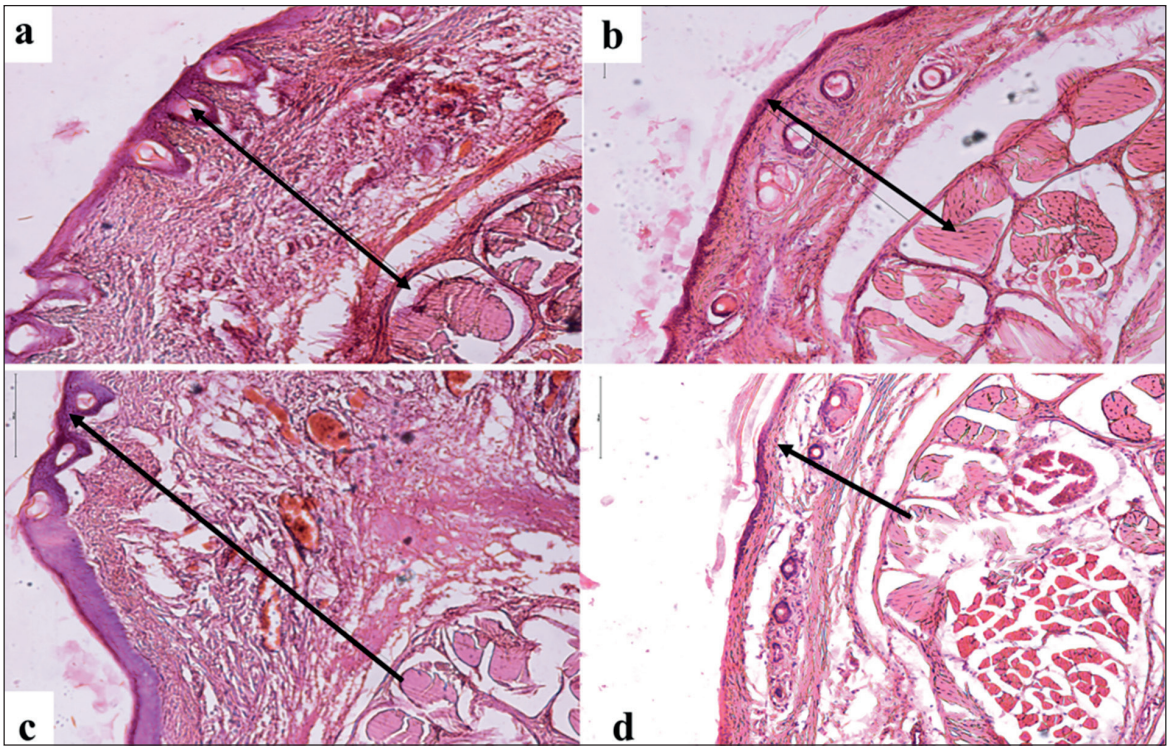


**Figure 10.** At day 5, W **(a)** control and **(b)** drug-treated mouse. At day 11, **(c)** control (highest swelling in the left area) and W **(d)** drug-treated mouse. The histological examination with hematoxylin/eosin staining of a cross section of a mouse tail in which the different thickness in the control tails compared to the treated tails is shown. The thickening in the dermal and hypodermic perivascular and perilymphatic layer is much higher in untreated mice than in treated mice. Furthermore, in untreated mice there was a marked inflammatory response, leukocyte infiltration and increased collagen deposition, compared to the treated mouse. (Magnification x10).

showing an improved phenotype[16,17,23]. Vitamin A from literature data was found to promote lymphangiogenesis and enhance lymphatic vessel regeneration[24]. Interestingly, in a previous work[20] we reported that patients with lymphedema can carry deleterious variants in the *RORC* gene, encoding the retinoid acid receptor-related orphan receptor C[20] and the levels of expression of the *RORC*-encoded protein were reported to be influenced by the administration of vitamin A[19]. Spermidine downregulates factors and pathways involved in the lymphedema-related inflammation, such as the NF-kB pathway[25,26], tumor necrosis factor-α, interleukin-1β, nitric oxide and prostaglandin E2[15].

The differences in tail swelling between control and DT groups were significant. Furthermore, a higher swelling degree in the first 5 days of treatment in the DT than in the control was observed, although this increment was not significant. This phenomenon might indirectly indicate that this drug is boosting the innate immune system, which is linked to higher swelling. This initial inflammation is a positive sign for better wound healing. Thereafter, on 6$^{th}$ day onward, there was a rapid and significant reduction in swelling. This shows promising therapeutic response of this drug for preventing the progress of lymphedema and its negative effects associated with inflammation. The third important finding was that the peak of swelling was anticipated to the 6$^{th}$ day in the DT group, whereas in the control group the peak was normally on the 10$^{th}$-11$^{th}$ day. Thus, higher increase of swelling in the early days of wound, followed by quick decrease of swelling, favours the process of natural wound healing. In fact, the sustained swelling observed in the control group delays the correct wound healing and causes a condition termed as chronic wound healing associated with longer times for recovering and higher risk of complications[27].

## Conclusions

Starting from the inflammatory pathways involved in lymphedema[7], our research allowed us to design formulation, apparently effective against lymphedema in the animal model, based on natural molecules with a possible synergistic effect. To date, our results are among the most promising in literature. The next step would be their confirmation in clinical studies.

**Conflict of Interest**
The Authors declare that they have no conflict of interests.

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