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A Preliminary Investigation of the Enzymatic Inhibition of 5α-Reductase and Growth of Prostatic Carcinoma Cell Line LNCap-FGC by Natural Astaxanthin and Saw Palmetto Lipid Extract *In Vitro*

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ABSTRACT. Inhibition of 5α-reductase has been reported to decrease the symptoms of benign prostate hyperplasia (BPH) and possibly inhibit or help treat prostate cancer. Saw Palmetto berry lipid extract (SPLE) is reported to inhibit 5α-reductase and decrease the clinical symptoms of BPH. Epidemiologic studies report that carotenoids such as lycopene may inhibit prostate cancer. In this investigation the effect of the carotenoid astaxanthin, and SPLE were examined for their effect on 5α-reductase inhibition as well as the growth of prostatic carcinoma cells *in vitro*. These studies support patent #6,277,417 B1.

The results show astaxanthin demonstrated 98% inhibition of 5α-reductase at 300 μg/mL *in vitro*. Alphastat, the combination of astaxanthin and SPLE, showed a 20% greater inhibition of 5α-reductase than SPLE alone *in vitro*. A nine day treatment of prostatic carcinoma cells with astaxanthin *in vitro* produced a 24% decrease in growth at 0.1 mcg/mL and a 38% decrease at 0.01 mcg/mL. SPLE showed a 34% decrease at 0.1 mcg/mL.

Conclusions: Low levels of carotenoid astaxanthin inhibit 5α-reductase and decrease the growth of human prostatic cancer cells *in vitro*. Astaxanthin added to SPLE shows greater inhibition of 5α-reductase than SPLE alone *in vitro*. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com> © 2005 by The Haworth Press, Inc. All rights reserved.]*

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KEYWORDS. Prostate, Saw Palmetto, astaxanthin

INTRODUCTION

Recent clinical evidence has shown that inhibition of the enzyme 5α-reductase can decrease the obstructive symptoms of benign prostate hyperplasia (BPH) as well as the size of the prostate.[1] Inhibiting 5α-reductase blocks the conversion of testosterone to 5α-dihydrotestosterone (DHT), a more potent androgen primarily responsible for prostate growth.[2] Recent *in vitro* studies report that Saw Palmetto (*Serenoa repens*) berry lipid extract (SPLE), to be a non-competitive inhibitor of 5α-reductase.[3,4] Subsequent clinical studies done to compare its efficacy to finasteride, a prescription 5α-reductase inhibitor. The results suggest they are equal effectiveness in treating the symptoms of BPH but SPLE had little effect on prostate specific antigen (PSA) or DHT levels.[1,5] These findings are significant because other research suggests DHT may be an important factor in the development of prostate cancer.[6] The combined conclusions of these studies suggest that a natural, potent 5α-reductase inhibitor with anticarcinogenic properties, would be extremely valuable for treating the symptoms of BPH and treating or possibly decreasing the risk of prostate cancer.

Recent epidemiologic studies have reported that carotenoids such as lycopene and beta-carotene may have properties important in the prevention or treatment of prostate cancer.[7,8] Astaxanthin, another dietary carotenoid and extremely potent antioxidant, from *Haematococcus pluvialis*, has also been reported to possess antitumorigenic and anticarcinogenic properties.[9] Its ability to inhibit or treat prostate cancer has not been reported. There are also no references pertaining to the effect(s) of carotenoids on 5α-reductase activity. Since inhibiting the conversion of testosterone to DHT may be a mechanism for prostate cancer, it was decided to evaluate this carotenoid *in vitro* as a basis for developing a single natural product for the prevention and/or treatment of both BPH and prostate cancer.

The purpose of this study is to examine the effect of astaxanthin (from *Haematococcus pluvialis*) alone, and combined with SPLE (Alphastat®) and SPLE on 5α-reductase activity *in vitro*. Also, to evaluate the effects astaxanthin and SPLE on the proliferation of human pros-

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tatic carcinoma cell line LNCap-FGC. These results support patent # 6,277,417 B1.

MATERIALS AND METHODS

Test Sample Preparation

SPLE: Dried berries from Saw Palmetto (*Serenoa repens*) were milled and extracted with alcohol. The alcohol was removed under vacuum resulting in an extract containing not less than 85% free fatty acids (Triarco Ind., Wayne, NJ).

Astaxanthin: Dried, milled algae meal (*Haemotococcus pluvialis*) containing 1.5-2% astaxanthin (Cyanotech Corporation, Kailua-Kona, Hawaii).

Alphastat: SPLE containing various amounts of astaxanthin meal (w/w).

5α-Reductase Inhibition Bioassays: In vitro assays to determine the effect of Astaxanthin, Alphastat, and SPLE on 5α-reductase were performed by Paracelsian Laboratories (Ithaca, NY), as described below.

Rat Liver Microsome Preparation: Isolated rat liver microsomes were the source of 5α-reductase. Sprague-Dawley rats (200-350 g) were euthanatized with CO_2. The liver was exposed and cut away from the portal vein. Perfusion buffer (HEDG Buffer: 25 mm HEPES, 1.5 mm EDTA, 1 mm DTT, 10% (w/w) Glycerol, pH 7.6 adjusted with HCl with 0.154 M KCl added) was dispensed through the portal vein until each lobe of the liver was fully perfused. Perfused liver was fully removed, rinsed with ice cold HEDG, weighed and homogenized in ice cold HEDG Buffer. The homogenates were pooled and centrifuged at 37,000 RPM for one hour at 4°C. Microsomes were reconstituted by adding 5 mL of HEDG buffer to each tube and resuspending the drained pellet. They were pooled, homogenized, dispensed in 400 mcL aliquots and stored in an ultra low freezer.

Inhibition Assay: The following mixtures were prepared in triplicate in 16 $\times$100 mm^2 glass test tubes and used to assay for biological activity: Potassium phosphate buffer (20 mM, pH 7.0), microsomes containing 5α-reductase and substrate testosterone in limiting concentration. The mixtures were challenged with a positive control (SPLE), astaxanthin, Alphastat or a negative control. The reaction is started by the addition of cofactor NADPH, incubated in a 37°C water bath for 60 minutes, removed and placed in an ice bath. The reaction was stopped

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by the addition of 3 mL of ether to each tube and mixing briefly. Each tube was spiked with 20 mcL of progesterone stock as an internal standard and mixed for 30 seconds. When the layers have separated the organic layer was transferred to a fresh tube. The extraction was repeated two more times using 3 mL ether and the organic phase pooled. The tubes were placed in an uncovered 50°C water bath for 10 minutes or until approximately one-half of the solvent evaporated, mixed for five seconds and placed back in the water bath until dry.

High Performance Liquid Chromatography (HPLC) Quantification of 5α-Reductase Activity: This assay quantifies testosterone as a result of the inhibition of 5α-reductase. The tubes prepared above were reconstituted with 530 mcL of methanol/tetrahydrofuran (75.5%:24.5%) and 470 mcL of deionized water. Each tube was analyzed in triplicate using an isocratic reversed phase HPLC system consisting of a 4 mm × 25 cm C18 column and ultraviolet detection at 240 nm. Quantification was performed using the peak area from direct injections of known concentrations of testosterone and progesterone reference standards. Results were considered acceptable only if the coefficient of variation was less than 20%. All statistical analyses to compare treatment groups were done with Microcal Origin version 5.0. Determinations of individual significant differences in 5α-reductase activity were performed relative to the control values using one-way ANOVA analysis. (Significant differences were defined as $p < 0.05$, $n = 3$.)

Human Prostate Cell Proliferation Assay

The cellular proliferation assay was conducted by NovaScreen® (Hanover, MD). The assay employed LNCaP-FGC (human prostatic cancer cells) and a proprietary green fluorescent dye (CyQuant®-GR) (Molecular Probes) which shows strong fluorescent enhancement when bound to cellular nucleic acids. Since cell proliferation is always accompanied by an increase in the absolute amount of nucleic acid this method can be used to quantify the amount of cell division. This fluorescent dye enables stable associations with nucleic acids which allows cells to be frozen and stored so treatments over several days can be assayed together to reduce experimental variation.

Cell Growth and Plating: Cells were grown for four days at 37°C and 5% CO_2 to confluence in RPMI 1640 + 2 mM l-glutamine + 10% fetal bovine serum (FBS) + 4.5 g/L glucose + 10% HEPES + 1 mM sodium pyruvate in a T175 flask (Costal). Cells are harvested by trypsinization

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and are then centrifuged at 600 × g and resuspended to a concentration of 4×10^{-3} cells/mL. Cells were checked for > 95% viability and then plated out in six 96 well plates (Costar) at a density of 2000 cells per well in 200 μL of RPMI 1640 + 2 mM l-glutamine +10% fetal bovine serum (FBS) + 4.5 g/L glucose +10% HEPES + 1 mM sodium pyruvate (growth medium).

Cell Treatment: Six plates were seeded at 2000 cells/well. One plate was removed, washed and frozen to serve as a baseline. Cell proliferation was followed for 9 days. Vancomycin (positive control) was prepared at 1×10^{-3} M, diluted with growth medium to a final concentration of 1×10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M in the wells. SPLE and astaxanthin were prepared and diluted to a final dilution of 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, and 1:1,000,000. All plates were then returned to a humidified incubator at 37°C and 5% CO_2 to continue growth. Plates were removed from the incubator on days 1, 2, 7, 8, and 9. The plates were then washed and frozen at − 70°C until assayed.

Proliferation Assay: The six plates (including the baseline plate) were removed from the − 70°C freezer and allowed to reach room temperature. The cell lysis buffer (molecular probes) was prepared by diluting the stock solution 20-fold in distilled water. Just prior to the assay the CyQuant-GR dye reagent was prepared by diluting the stock solution diluted 800-fold with 1 × lysis buffer. Each of the 96 well plates contained 1.5 mL distilled water, 500 μL cell lysis buffer and 100 mcL CyQuant-GR dye reagent. The plates were agitated and incubated for 10 minutes at room temperature in the dark. Fluorescence was quantified on a Victor2 fluorometer (Perkin-Elmer, Boston, MA) at excitation wavelength (λ) of 485 nm and emission λ of 535 nm. All test compound data were reported as a percent (%) of total control wells on the same plate. A standard curve of bacteriophage λ phage DNA was performed on a separate plate in duplicate on the day of the assay. All statistical analyses to compare treatment groups were done with SPSS for Windows version 9.0. The effect of both treatments on cell growth was assessed by a general linear model multivariate analysis. Determinations of individual significant differences in growth and exposure time were performed relative to the same day control levels using a post-hoc Tukey test. (Significant differences were defined as $p < 0.05$, $n = 4$.)

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RESULTS

The results of the 5α-reductase inhibition assay in Table 1 show that astaxanthin demonstrated 98% inhibition at a concentration of 300 μg/mL. Since the level of pure astaxanthin in the algae meal was 1.5-2%, the final concentration of the pure carotenoid was approximately 4.5 to 6 mcg/mL. Alphastat 2.5 (SPLE + 2.5% w/w astaxanthin) produced an approximate 23% increase in inhibition over the SPLE control. Statistical testing showed that this is a significant inhibition. Alphastat 5 and Alphastat 10 (SPLE + 5% and 10% astaxanthin) did not significantly increase inhibition more than 23%. Alphastat 1 and 0.5 (SPLE + 1% and 0.5% astaxanthin) did not produce an inhibition significantly greater than the SPLE control.

The results of the prostatic carcinoma cell LNCap-FGC growth inhibition assay are shown in Table 2. After nine days of treatment with various levels of astaxanthin, statistically significant decrease in growth were obtained at the 0.1 mcg/mL (10^{-4} mg/mL) level and at the 0.01 mcg/mL (10^{-5} mg/mL) level. The results of Table 3 show a significant inhibition in the growth at an SPLE dose level of 0.1 mcg/mL (10^{-4} mg/mL). Multivariate analysis of the effects of saw palmetto ($F = 20.6$, $df = 5$), and

TABLE 1. The Results of *In Vitro* 5α-Reductase Inhibition Assays on a SPLE (Saw Palmetto Lipid Extract) Control, Astaxanthin (algae meal–*Haemotococcus pluvialis*) Containing 1.5-2% Astaxanthin and Alphastat (SPLE containing various amounts of astaxanthin, w/w). The Placebo Contained No LESP or Astaxanthin.

Sample Tested (50 μg/mL)	Average (n = 3) 5α-Reductase Inhibition	Relative Standard Deviation
SPLE Control	35%	2.8%
Astaxanthin, 300 mcg/mL	98%	2.0%
Alphastat 10 (SPLE + 10% w/w astaxanthin)	43%	1.7%
Alphastat 5 (SPLE + 5% w/w astaxanthin)	43%	1.4%
Alphastat 2.5 (SPLE + 2.5% w/w astaxanthin)	42%	1.8%
Alphastat 1 (SPLE + w/w astaxanthin)	34%	2.7%
Alphastat 0.5 (SPLE + 0.5% w/w astaxanthin)	34%	2.3%
Placebo	1.2%	1%

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TABLE 2. Nine Day Treatment of Prostatic Carcinoma Cell–LNCap-FGC with *Haematococcus pluvialis* Algae Meal Containing 1.5-2% of the Carotenoid Astaxanthin. Inhibition of Growth Is Calculated as Percent Reduction from Control Culture (cells grown with media alone) Levels. *p < 0.01.

Percent Growth Inhibition of LNCap-FGC Prostatic Carcinoma Cells Treated with Astaxanthin						
Day	Percent Inhibition of Growth					
	10^{-9} mg/mL	10^{-8} mg/mL	10^{-7} mg/mL	10^{-6} mg/mL	10^{-5} mg/mL	10^{-4} mg/mL
0	−12	−5	4	5	−1	−3
1	−5	−4	4	−4	11	27*
2	−4	−1	−3	−5	20*	22*
7	4	−2	−2	−1	21*	26*
8	−2	0	−1	−3	32*	19*
9	−2	−3	−1	−3	38*	24*

TABLE 3. Nine Day Treatment of Prostatic Carcinoma Cell–LNCap-FGC with Saw Palmetto Lipid Extract (LESP). Inhibition of Growth Is Calculated as Percent Reduction from Control Culture (cells grown with media alone) levels. *p < 0.01.

Percent Growth Inhibition of LNCap-FGC Prostatic Carcinoma Cells Treated with Astaxanthin						
Day	Percent Inhibition of Growth					
	10^{-9} mg/mL	10^{-8} mg/mL	10^{-7} mg/mL	10^{-6} mg/mL	10^{-5} mg/mL	10^{-4} mg/mL
0	−8	−8	1	0	8	−1
1	0	−1	6	−8	−6	34*
2	−9	9	2	2	−6	28*
7	−8	−2	0	−1	−7	48*
8	−5	1	−4	−4	−3	42*
9	−7	−2	−5	−8	−9	34*

astaxanthin ($F = 46.3$, $df = 5$) showed a significant effect of compound concentration, but not days of exposure. Significant interactions between exposure and concentration were also seen with both compounds.

DISCUSSION

The present study reports astaxanthin (from *Haematococcus pluvialis*) algae meal to be a potent inhibitor of 5α-reductase *in vitro*. It produced a

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98% inhibition at a concentration of 300 mcg/mL. Since the level of pure astaxanthin was 1.5-2%, the final concentration of the pure carotenoid was approximately 4.5 to 6 mcg/mL. Proportionally, approximately 144 mcg/mL of SPLE would have to be used to produce an equivalent inhibition. This indicates that astaxanthin may be 24 to 32 times more potent as an inhibitor of 5α-reductase than SPLE and would appear to explain the significant increase in inhibition produced by Alphastat. The lack of a linear increase in inhibition at more than 2.5% (w/w) astaxanthin, may be due to the SPLE becoming saturated with the lipid soluble astaxanthin carotenoid.

A search of the literature found no references to the ability of astaxanthin-type carotenoids to inhibit 5α-reductase enzymes. The inhibition therefore, was certainly unexpected and is the basis of patent # 6,277,417 B1. At this time, the mechanism is unclear but may be similar to that of SPLE. Recent *in vitro* studies suggest that the lipid component of SPLE may be responsible for its inhibitory effect by modulating the membrane environment of 5α-reductase.[4] Since astaxanthin is also very lipid soluble, it may have a similar mechanism. More specifically, its lipophilic properties allow it to become associated with cellular membranes by exerting Itself between lipid bilayers.[10,11] Since 5α-reductase is a membrane-associated enzyme and highly sensitive to membrane composition, it can be stimulated or inhibited by the presence of specific membrane components.[4] Regardless of the mechanism, the data presented here suggests that the inhibition of 5α-reductase by astaxanthin could be important for decreasing the symptoms of BPH.

There are several reports that astaxanthin provides protection to a range of cancers including colon, bladder and oral by the suppression of cell proliferation.[12-14] From this it may be inferred that astaxanthin might reduce the risk or help in the treatment of prostatic cancer. This prompted the investigation of the effect of astaxanthin on prostatic carcinoma cell LNCap-FGC growth. SPLE was also tested since it is a known inhibitor of 5α-reductase. The results in Table 2, showing astaxanthin inhibits the growth of this cell line was somewhat unexpected. Even more unexpected was to get a 38% inhibition (day 9) at a level of 0.01 mcg/mL. Since the meal is 1.5 to 2% astaxanthin, this would be equivalent to 0.00015 to 0.0002 mcg/mL astaxanthin. Inhibition of cell growth at this level *in vitro* suggests that the recommended dose of 2 mg of the carotenoid astaxanthin per day may be enough to inhibit prostatic carcinoma cell growth *in vivo*.

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Another unexpected result of this research is that SPLE had some inhibitory effect on the growth LNCap-FGC. The results in Table 3 show 34% inhibition in growth at 0.1 mcg/mL SPLE. This percent inhibition is approximately equivalent to 1000 × the dose of astaxanthin. The results suggest that perhaps the daily dose SPLE should be 2000 mg to support prostate health rather than 320 mg which is currently recommended.[1]

CONCLUSIONS

Low levels of carotenoid astaxanthin, from *Haematococcus pluvialis* algae meal, and astaxanthin combined with SPLE inhibit 5α-reductase. Astaxanthin added to SPLE shows greater inhibition of 5α-reductase than SPLE alone *in vitro*. Astaxanthin and SPLE decrease the growth of human prostatic cancer cells *in vitro* but appear to be very different in potency.

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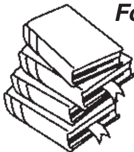

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