Biochemical Characterization of Lycopene Extracted from *Citrullus Lanatus* and its Therapeutic Potential to Dalton’s Lymphoma Ascites Tumor Model

G. Renuga¹, R. Sharmila²

¹Principal & Research Co-ordinator, Sri Adi Chunchanagiri Women’s College,
²R. Sharmila, Research Scholar, Dept of Biochemistry, Sri Adi Chunchanagiri women’s College, Cumbum, Theni Dr, Affiliated to Mother Teresa Women’s University, Tamil Nadu, India.

Corresponding Author: G. Renuga

ABSTRACT

**Background and purpose:** Cancer is a major public health burden in both developed and developing countries. Natural and some synthetic compounds can prevent, suppress, or reverse the progression of cancer. The main purpose of the work is to provide cheapest plant based therapy for treatment of cancer, in order to reduce the risk of cancer.

**Material and Methods:** Bioactive compound namely lycopene was extracted from *Citrullus lanatus* fruit and its purity was analyzed by HPLC. The tumor induced swiss albino mice was treated with lycopene and comparative studies was carried out with standard available carboplatin. Experimental samples/tissues of the mice were subjected to various experiments to find out efficacy of lycopene action against Dalton’s lymphoma ascites tumor. Impact of lycopene in cancer expressed in terms of suppression in cell arrest/control measures were confirmed by western blot techniques

**Result:** Lycopene combined carboplatin showed promising cytotoxic activity against Dalton’s lymphoma ascites tumor when compared to carboplatin. The experimental research showed better healing or suppression of cancer proliferation.

**Conclusion:** Experiment was designed in the form of cancer induction in mice model, different batches of experimental animals were treated and results concluded that combination of carboplatin with lycopene showed better therapeutic effect on Dalton’s lymphoma ascites tumor. In future lycopene will be formulated as pharmaceutical product.

**Keywords:** Antioxidant, *Citrullus lanatus*, Dalton’s lymphoma ascites, HPLC, Lycopene,

INTRODUCTION

Researchers are focusing on exploitation of natural resources for dietary regimen against life threatening ailments. [1] Plant based diet contains several bioactive ingredients with vital role to perform various metabolic functions like growth, development and protective mechanism against physiological threats. In this context, phytochemicals are of significance as improve the human health through distinct pathways.

Watermelon (*Citrullus lanatus*), botanically considered as a fruit, belongs to the family *Cucurbitaceae*. [2] It is native to Kalahari Desert of Africa but nowadays, it is also cultivated in tropical regions of the world. Fruit of this plant is major consumed portion and variations in growth characteristics determine its quality. [3] Considering the nutritional profile, consumption of 100 g watermelon provides 30 kcal. It contains almost 92 % water and 7.55 % of carbohydrates out of which 6.2 % are sugars and 0.4 % dietary fiber. It is
enriched with carotenoid, vitamin C, citrulline, carotenoids and flavonoids and fat and cholesterol free, thus considered as low caloric fruit. [4] Additionally, watermelon is rich source of β-carotene acts as an antioxidant and precursor of vitamin A. Besides the presence of lycopene, it is a source of B vitamins, especially B₁ and B₆, as well as minerals such as potassium and magnesium. [5]

Earlier, only tomato and its products were considered as potential sources of lycopene but now there are proven facts that watermelon also contains appreciable amount of cis-configured lycopene. Thus consumer is gradually shifting towards watermelon and its allied products for their health concerns. A complex mechanism persists in the biosynthesis of lycopene that starts when chlorophyll degrades to yield white colored leucoplast thus yielding specialized red color pigmented organelles i.e. chromoplast. [6]

Presently, a number of evidences are available indicating direct linkages between food active components and cell genomic with special reference to cancer treatment. Nutrigenomics is a broader term that explains interaction of nutrient with gene expression. Being active dietary component, lycopene interferes at various stages of cancer development i.e. DNA mutation and tumor metastasis thus have direct impact on gene and inhibit mutation. [7] However, understanding of lycopene and gene interaction has not yet been well established and needs further research. Lycopene is likely to be associated in the production of phase I and II enzymes that are essential for metabolism of carcinogen within the physiological system.

Generally, these enzymes work in harmony but in case of ROS over production, interruption may occur resulting necrosis or apoptosis. In such cases, dietary lycopene acts as a therapeutic agent to combat excessive ROS production.

Recent studies suggest that the dietary intake of Watermelon and its products containing lycopene are associated with a decreased risk of cardiovascular disease. In order to substantiate these facts, the present study was undertaken to investigate the effectiveness of lycopene from watermelon products on the potential effects of oxidative stress and cancer, focusing on early atherosclerotic events. The present study was planned to evaluate the macromolecules and micronutrients potentials, the phytochemical components, antioxidant activity of solvent extract of the selected Citrullus lanatus fruit (Watermelon) belonging to family Cucurbitaceae, and experiments were carried out for characterization of lycopene and standardize the novel purified methodology subsequently an attempt was made to find out the role of lycopene in Dalton's lymphoma ascites tumor induced in mice model.

GST inducers are generally considered as protect on compounds against cancer, acting as blocking agents. An increase in GST activity may induce a general protection state, leading to an inhibition in cancer tissues/cells. Therefore, it was interest to determine GSH levels in various tissues during ascites Dalton’s lymphoma growth in vivo and carboplatin treatment. An attempt was made to identify the mechanism of GST changes and its significance in combination of carboplatin and lycopene mediated cancer chemotherapy. Present study was designed in order to evaluate administration of purified lycopene combined with carboplatin can be carried out the dose response of induced changes in the endogenous antioxidant defense system such as GSH level, antioxidant enzyme activities of glutathione-S-transferase (GST) in the tumor bearing mice.

**MATERIALS AND METHODS**

**Extraction of Lycopene:**

*Citrullus lanatus* (Watermelon) fruits were collected from in and around Theni. Lycopene from *Citrullus lanatus* was extracted as follows: 20g of sample (red pulp) was homogenized in 100mg calcium
bicarbonate and 70-80% warm ethanol and filtered using Whatmann no.1 and no.42 filter paper. Again the residue was homogenised with 95% warm ethanol and filtered using Whatmann no.1 and no.42 filter paper. The ethanol solution was recovered and centrifuged at 5000 rpm for 10 minutes and the supernatant was discarded and small amount of double distilled water was added to the residue again centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the residue was dried in room temperature to obtain the final product lycopene crystals.

Obtained lycopene crystal was dissolved in a sample of hexane: acetone (1:1, v/v), and quantified spectrophotometrically at 472 nm and expressed in mg/100 g FW. Samples were placed in 15 ml polypipilene containers, and stored at -20°C until further analysis. Standard of lycopene was purchased from Sigma-Aldrich Chemical Co.

**Analysis of pharmaceutical potentials:**

Phytochemical screening of extracted sample were carried out according to the standard methods [9,10] for alkaloids, tannins, flavonoids, steroids, glycoside, phenol, saponins, terpenoids, and quinons.

**Spectral analysis:** Primary identification test performed using colour chemical reactions and identification of chemical structure of the isolated lycopene was done using, NMR.

**Determination of compounds by HPLC:**

Samples were extracted as above mentioned protocol and amount of aromatic compounds were determined with HPLC. Spent medium was extracted with ethyl acetate, evaporated and extracts were analyzed by HPLC. Chromatographic analysis was performed with a liquid chromatography equipped with a LCP 4100 solvent delivery system, an AS 54 autosampler, a LCO 101 column oven, and a LCD 2084 variable wavelength spectrophotometric detector. Data were collected digitally using Clarity chromatography software (DataApex, Prague, Czech Republic). Spectrophotometric analyses were carried out on a UV-1700 Pharma Spec spectrophotometer. [11,12]

**Experimental animal maintenance:**

Inbred Swiss Albino mice (20-25g) were maintained in the Laboratory Animal Feeds, (Bangalore) and albino mice were maintained in the laboratory with free access to commercially available food pellets and water. For each experimental group 4-5 mice aged 10-12 weeks (25-30g) were used. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions [Temperature 27°C and 12 hours light/dark cycle] throughout the experimental period. Animal experiments were carried out following the guidelines of the animal ethics committee of the Institute. Dalton’s Lymphoma ascites was obtained from Amala Cancer Institute, Trisshur, Kerala Dt.

**Tumour Induction:**

Ascites Dalton’s lymphoma tumor was maintained in vivo by intraperitoneal (ip) transplantation of 1X10^6 tumor cells per animal (0.25 volumes, in phosphate buffered saline, PBS). PBS was prepared by adding 0.15 M NaCl to 0.01 M sodium phosphate buffer, pH 7.4. Since tumor-transplanted animals usually survived for 18-20 days, and were allowed to grow with tumor. The treatment was started from the 2nd and 11th day for the experimental design and 3,5 and 10 days were considered to be the initial, middle and later stages of tumor growth, respectively.

**Experimental scheduled given below:**

Experimental animals were divided into three treatment groups involving 60 mice of 4 batches containing 6 mice each and injected as follows:

Batch 1 animals received normal saline and were used as control (saline 10ml /kg of body wt intraperitoneal (i.p) injection (n=5). Batch 2 animals were induced cancer with Dalton’s lymphoma ascites (Amala cancer Institute Trissur, Kerala). Ascites Dalton’s
lymphoma tumor was maintained in vivo by intraperitoneal (ip) transplantation of 1 x 10^6 tumor cells per animal (0.25 volume, in Phosphate-buffered Saline, PBS). PBS was prepared by adding 0.15 M NaCl to 0.01 M Sodium phosphate-buffered pH 7.4 animals were allowed to grow with tumor. Treatment was started from the 2nd and 11th day for the batch 2 served as normal. Batch 3 animals received a single dose of drug carboplatin was administrated to tumor bearing mice on the 8th day of tumor growth (maintained as experimental group 1). (Preparation of doses: Carboplatin Dose (10 mg /ml was dissolved in 5% dextrose in water and use immediately).

Batch 4 animals received a combination dose of lycopene 100µg/kg body wt,i.p) was administered to tumor bearing mice before 8 h of sub- therapeutic dose of carboplatin (5 mg /kg body wt,i.p) administration (maintained as experimental group 2). Treatment was started 24 hrs after the tumor inoculation and continued for 11th days, body weight of animals were noted daily in all groups during treatment period. Animal were kept to check the survival time of DLA bearing mice, subsequently animals were sacrificed by anesthesia and organs tissue were isolated (liver, blood, ascites tumor) frozen in liquid nitrogen and stored at - 80°C until biochemical analysis could be completed. If any death, of the animals in different groups were recorded daily and the survival pattern of the animals were determined for different group. The parameters such as survival time, packed cell volume, body weight were studied during the period of experiment.

**Measure of Viability:** This procedure can be performed along with the cell counting procedure but cell density may require adjustment in order to obtain approximately 10^6 cells per milliliter. Mix 1 drop of trypan blue with one drop of the cell suspension and allow 1 - 2 minutes for absorption. Prepare haemocytometer and load chambers as described in "Cell Quantitation". Count both the total number of cells and the number of stained (dark) cells

**Calculation:** percent viability= (Total cell counted –Stained cells) X 100 / Total cells counted

The ascites tumor cells are obtained by aspirate with Phosphate buffer saline in the peritoneal Cavity of DLA bearing mice. The cells were then mixed with 0.4% Trypan blue in the ratio of 1:1 and the cells were counted using the Haemocytometer. (Live cells do not take stain whereas the dead cells get stained)

**Western blotting:** Liver tissues crude extract (40µg protein per lane) were analyzed by 15% SDS-PAGE. [13] Proteins were transferred electrophoretically to nitrocellulose filters (for 3 h at1A) using an immunoblot transfer apparatus. After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3% (w/v) BSA in Tris-buffered saline (TBS; 500mM NaCl and 20mM Tris-HCl pH 7.5) to block non specific binding. The blot was incubated overnight at 4°C with 3% (w/v) BSA in TBS containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.1% BSA and 0.2% Nonidet P40, the blot was incubated for 1 h at room temperature with peroxidase- conjugated goat anti (mouse immunoglobulin) diluted at 1:100 in 3% BSA and 0.2% Nonidet P40. Antibodies were visualized using a chem.-illuminescence detection system.

**RESULTS AND DISCUSSION**

In commercial plant *Citrullus lanatus* reliable source Lycopene, the red pigment which is a lipophilic compound that is insoluble in water, but soluble in organic solvents, and it has a quenching constant double that of beta-carotene and 10 times alpha tocopherol (Hadley and others 2003). The extraction and isolation of lycopene has been carried out using different methods and the yield of lycopene showed to be high in natural sources of lycopene (Figure 1). The benzene test initially helps us to identify
lycopene in the residue. A simple liquid-liquid extraction method was employed to extract lycopene in minimum organic solvent. Crystals were purified by recrystallization from ether. Obtained crystals were then observed under projection microscope. Figure 2 showed concentrated and air spray dried at low temperature by spray drying technology which captures red color, pure taste and rich lycopene of ripe watermelon. Lycopene is a member of carotenoid family, is a lipid soluble antioxidant synthesized by many plants and microorganisms but not by animals and humans where it serves as an accessory light-gathering pigment and protects them against the toxic effects of oxygen and light. It is a red pigment without provitamin-A activity that imparts colour to many fruits and vegetables.

**Phytochemical analysis:**

Methanol extract of *Citrullus lanatus* has screened to identify compound of therapeutic potential source for the drugs against human illnesses. Extract of *Citrullus lanatus* were contributed different compounds. The methanolic extract of *Citrullus lanatus* table 1 shows the presences of secondary metabolites like flavanoids, glycosides, saponins, phenol, tannin, steroids, diterpenes and absence of alkaloids, aminoacids, anthraquinones.
Table 1: Phytochemical Analysis

<table>
<thead>
<tr>
<th>S.No</th>
<th>Secondary metabolites</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Amino acid</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Diterpenes</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Anthraquinones</td>
<td>-</td>
</tr>
</tbody>
</table>

(+ indicates presences, - indicates absences)

HPLC Chromatogram

The mobile phase was a mixture of methanol and ethanol (75 : 25, v/v). The flow rate was kept constant at 0.8 ml/min. Optimum response of β-carotene at 450 nm, and lycopene at 468 nm. The amount of individual component was quantified from the corresponding peak area ratio of internal standard β-carotene and lycopene as sample using Clarity chromatography software (DataApex). The concentration of each analyte in the samples was determined from its calibration curve. It is very important that extracts were to be dried as quickly as possible, especially lycopene, are not stable in n-hexane that stored at −80°C are stable for at least one year. It is necessary that all procedures were to be performed in a darkened room. Several eluents (mixtures of organic solvents such as acetonitrile, methanol, ethanol, 1-propanol, 2-propanol, and n-hexane) and several gradients were assessed. The best results were obtained for the conditions that described in “Chromatographic Analysis.” Column temperature was changed from 25 to 45°C. Optimal temperature interval was from 40 to 45°C. The criteria were resolution, stability of the absorbance, and analysis duration. According to results, presented method is highly efficient for chromatograms of lycopene in standard solution for HPLC as purity 87.7–95.8% for lycopene.

Fourier transforms IR spectroscopy (FT-IR). Standard spectra were collected by using a Perkin Elmer Spectrum GX spectrometer with a potassium bromide beam splitter and deuterated triglycerine sulfate (DTGS) detector were used for all readings. A range of 400–4000 scans were accumulated for each spectrum at a spectral resolution of 4cm⁻¹. It was possible to use the drift accessory with the powdered pure substance, thereby allowing for a better and easier analysis. Regarding the image in visible absorption spectra for samples analyzed makes the difference between them is the ratio of the maximum absorption is closely related to the composition of carotenoid extracts. Lycopene is the most important contribution to the absorption band at 470 nm and 500 nm.

Table 2: Precision of lycopene of β-carotene in Citrullus lanatus:

<table>
<thead>
<tr>
<th></th>
<th>Lycopene µmol/ml</th>
<th>Mean±SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A- Precision (with in the day)</td>
<td>0.069 ± 0.005</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>B- Precision (between the day)</td>
<td>0.096 ± 0.007</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.099 ± 0.009</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Average parameters of 10 calibration curves for HPLC method:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression Equation</th>
<th>Mean slope 95% confidence interval</th>
<th>Intercept 95% confidence interval</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene1</td>
<td>Y=1.375x+0.0020</td>
<td>1.3854(1.2731-1.4001)</td>
<td>-0.001(-0.005 - 0.003)</td>
<td>0.9983</td>
</tr>
</tbody>
</table>

Legend for table 3: Nine point for the determination of analytical parameters and seven point for routine analysis. The x intercept (in µmol/L) is the point at which the line crosses the x-axis (where the y value equals 0).

By using both analytical techniques, was possible to determine lycopene from watermelon samples. Table 3 showed the average predicted IR lycopene content of sample breeding lines as compared to the HPLC reference values. Therefore, the comparison between the results obtained by standard HPLC and the proposed FTIR methods indicates that the two procedures give statistically comparable values of lycopene concentration in watermelon samples. The accuracy of the proposed and standard methods was good.

The retention time for lycopene detected by HPLC has a specific value both in standard and in powder tomatoes, as it is shown in Figure 5. A reliable, selective, and sensitive HPLC method with spectrophotometric detection for the determination of lycopene, in watermelon sample was developed. Proper sample preparation preventing fat-soluble antioxidant vitamins degradation is required to achieve accurate analyses. The crucial points are sample collection, protein precipitation, and liquid-liquid extraction. The best results were obtained under these conditions: Stock solutions of β-carotene (∼20 mg/L; 37 µmol/L), lycopene (∼20 mg/L; 37 µmol/L), concentration of β-carotene = A * 4.74 (µmol/L) at 450 nm; concentration of lycopene = A * 3.56 (µmol/L) at 468 nm.

Table 4: Percentage of Tumor inhibition

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tumor induced as normal</th>
<th>Carboplatin drug treated</th>
<th>Carboplatin plus Lycopene treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed volume</td>
<td>6.7 ± 0.21</td>
<td>3.9 ± 0.30</td>
<td>2.7 ± 0.23</td>
</tr>
<tr>
<td>% Tumor inhibitions</td>
<td>0 %</td>
<td>35.09 %</td>
<td>57.14 %</td>
</tr>
</tbody>
</table>

Legends for table 4: The data illustrate the results of an experimental set up that was repeated three times. Mean ± SD of 5 Experiments.

Table 4 revealed the result of percentage increased in life span (% ILS). The results were as expected that carboplatin and combination of carboplatin drug with vitamin have shown sufficient cytotoxicity with trypan blue dye exclusion techniques as shown in Table 1. The efficiency of the drug administration was studied and the results shown that the Carboplatin plus vitamin treated group was found to be more effective and in exhibiting the antitumor activity against DLA cells.

Figure 6: Analysis of GST expression by Western blot

Legend for figure 6: Protein samples were prepared from experimental animals and analyzed by western blotting techniques; the GST expression was observed as image shown: Lane 1. GST molecular weight markers (36.5–55.4 kDa); Lane 2. GST specified protein from normal mic, Lane 3. GST specified protein from tumor induced, Lane 4. GST specified protein from tumor treated with carboplain, Lane 5. GST specified protein from tumor treated with carboplain plus Lycopene.
Angiogenesis plays a very important role in the growth of a tumor cell. This is because the tumor cell just like normal cells requires constant supply of oxygen and nutrients to survive and progress to infect other organs. This background knowledge of tumoral angiogenesis has created the right platform for scientists to come out with therapeutic methods that target the inhibition of tumoral angiogenesis. An effective method that inhibits tumoral angiogenesis will cut supply of oxygen and nutrients to tumor cells and therefore prevent their growth and metastasis. Present work have been able to identified inhibitors to tumoral angiogenesis, Moreover, the nonspecific inhibition on VEGF induced downstream pathways that brought about some side effects such as inhibition on growth or cell cycle arrest indirectly correlate with VEGF expression particularly on hypertension and endothelial bleeding. However we believe the development of novel compound based drug against VEGF will offer a promising solution to cancer therapies.

Nevertheless, the quantity of lycopene varies depending upon the variety and growing conditions. Overall, lycopene ranges from 2.30-7.20 mg/100 g fresh weight bases, present in crystalline form in cell. More interestingly, lycopene contents of red fleshed watermelon are almost 40 % higher than tomato i.e. 4.81 and 3.03 mg/100 g, respectively. However, yellow orange and yellow colored fleshed have relatively less lycopene content i.e. 3.68 and 2.51 mg/100 g, respectively.

Lycopene is a carotenoid that is produced as an intermediate product of xanthophylls production; β-cryptoxanthin, zeaxanthin, leutin etc. Carotenoids are basically formed by 40-C isoprenoids (5-C isoprene unit), called tetraprenoids. A stepwise addition of isopentenyl diphosphate (IPP) takes place with dimethylallyl diphosphate (DMAPP) giving rise 20-C precursor, geranylgeranyl diphosphate (GGPP). On desaturation of GGPP, 11 conjugated double bonds are produced that exist as lycopene in nature.

Lycopene has potential to prevent various chronic ailments like dyslipidemia, diabetes, oncogenesis, neurodegenerative diseases, osteoporosis etc. The protective aspects are ascribed to the singlet oxygen scavenging ability. Numerous metabolic syndromes arise due to high free radicals formation reacting with macromolecules thus oxidizing proteins, lipids and DNA. Lycopene protects humans from various pathogenic attacks responsible for an array of diseases. Several authors have reported that lycopene holds nutraceutical potential and being antioxidant provides protection against free radicals and oxidative damage. Free radicals are produced in the body during oxidation reduction reaction. However, excessive production deteriorates body defense mechanism, cell membrane and organelles. Although it has been used as a food colourant for many years, it has recently received attention with respect to its antioxidant activity and potential in preventing cancer and cardiovascular diseases in humans.

A typical carotenoid such as lycopene displays maximum absorbance at 476nm. Spectrophotometry results revealed that lycopene showed maximum absorbance at 476nm, followed closely by absorbance at 503nm. The purified lycopene content was found to be the maximum in watermelon (6.54 mg/kg). Results of the studies showed that the fruits analyzed having high concentration of Lycopene.

**CONCLUSIONS**

*Citrullus lanatus* can be a good natural source of lycopene which are naturally occurring substances found in many plants. Carotenoids in general have undergone a number of research studies as to their possible benefits against diseases, among other health issues. Lycopene is considered as antioxidant and anticancer agents while lycopene showed marked effects on dalton’s Lymphoma ascites tumor.
in animal model. When Carboplatin enters the cells, it is potentially vulnerable to cytoplasmic inactivation by these and other intracellular components. Elevated levels of GST are associated with increased resistance to apoptosis initiated by a variety of stimuli and it was plausible that GSTs serve via direct detoxification as well as acting as an inhibitor of the MAP kinase pathway. The use of therapeutic dose of carboplatin in combination with lycopene has been suggested to have better therapeutic efficacy than carboplatin alone, and decreased side effects in the host. Although promising data from epidemiological, as well as cell culture studies suggest that lycopene and the consumption of lycopene containing foods may affect cancer or cardiovascular disease risk, more clinical trial data is needed to support this hypothesis. In addition, future studies are required to understand the mechanism(s) whereby lycopene or its metabolites are proven to possess pharmaceutical activity against cancer cells. The present work confirms current knowledge with respect to its role in human health.

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