



IJPPR

INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH  
An official Publication of Human Journals

ISSN 2349-7203




Human Journals

Research Article

September 2016 Vol.:7, Issue:2


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# Characterization of Bioactive Molecule Lycopene from Water Melon and Evaluation of *In Vitro* Cytotoxic Activity against Human Colorectal Adenocarcinoma Cell HT-29



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ISSN 2349-7203



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**Submission:** 29 August 2016  
**Accepted:** 5 September 2016  
**Published:** 25 September 2016

**Keywords:** Carotenoids, Lycopene, Cytotoxic activity, IC<sub>50</sub>

## ABSTRACT

**Abstract:** The main aim of the present research work is to isolate the carotenoid lycopene from the ethanolic extract of Water melon (EEWM) and evaluate the cytotoxic activity against HT-29 cell line. The isolation of lycopene from EEWM was carried out by UV, IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, Mass spectra, and HPLC. The cytotoxic potentiality of EEWM was performed by SRB assay against human colorectal adenocarcinoma HT29 cell line. The results obtained from the *in-vitro* studies displayed that the ethanolic extracts of the fruit of *Citrullus vulgaris* L. (EEWM) possessed a very good cytotoxic activity against human colorectal adenocarcinoma HT-29 cell line. From the present experimental data it had been concluded that EEWM was exhibiting the potential capability to inhibit the growth of cancer cell when compared with standard drug 5-FU and the cell growth inhibition of EEWM was found to be the highest 92.95% at 10 µg (IC<sub>50</sub> = 2.7 µg/ml). The IC<sub>50</sub> value of standard drug 5-FU was found to be 1.399 µg/ml with 95.97 % growth inhibition at concentration 75 µg/ml.



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## INTRODUCTION [1, 2, 3]

The watermelon (*Citrullus vulgaris*, family Cucurbitaceae) is an annual plant with long, weak, trailing or climbing stems which are five-angled and up to 3 m (10 ft) long. Young growth is densely woolly with yellowish-brown hairs which disappear as the plant ages. The leaves are stemmed and are alternate, large and pinnately-lobed, stiff and rough when old. The plant has branching tendrils. The flowers grow singly in the leaf axils and the corolla is white or yellow inside and greenish-yellow on the outside. The flowers are unisexual, with male and female flowers occurring on the same plant (monoecious). The male flowers predominate at the beginning of the season and the female flowers, which develop later, have inferior ovaries. The styles are united into a single column and the large fruit is a kind of modified berry called a pepo. This has a thick rind (exocarp) and fleshy center (mesocarp and endocarp). Wild plants have fruits up to 20 cm (8 in) in diameter while cultivated varieties may exceed 60 cm (24 in). The rind of this fruit is mid- to dark green and usually mottled or striped, and the flesh contains numerous pips and is red, orange, pink, yellow, green or white.



**Fig-1: Watermelon plant**



**Fig-2: Watermelon**

## HEALTH BENEFITS [4, 5]

Watermelons are mostly water about 92 percent but this refreshing fruit is soaked with nutrients. Each juicy bite has significant levels of vitamins A, B6 and C, lots of lycopene, antioxidants, and amino acids. There's even a modest amount of potassium. Plus, this quintessential summer snack is fat-free, very low in sodium and has only 40 calories per cup. Watermelon's high levels of lycopene are very effective at protecting cells from damage and may help lower the risk of heart disease, according to a study at Purdue University. Also, the fruit's concentrations of citrulline and arginine are good for your heart. Arginine can help improve blood flow and may help reduce the accumulation of excess fat. A study published in the American Journal of Hypertension found that watermelon extracts helped reduce hypertension and lower blood pressure in obese adults.

**Hydration:** "Watermelons are the perfect example of a food that can help you stay hydrated," said Jarzabkowski. Their water content can help keep you hydrated, and their juice is full of good electrolytes. This can even help prevent heat stroke.

**Digestion:** The watermelon contains fiber, which encourages a healthy digestive tract and helps keep you regular.

**Skin and hair benefits:** Vitamin A is stellar for your skin, and just a cup of watermelon contains nearly one-quarter of your daily recommended intake of it. Vitamin A helps keep skin and hair moisturized, and it also encourages healthy growth of new collagen and elastin cells, according to the Cleveland Clinic. Vitamin C is also beneficial in this regard, as it promotes healthy collagen growth.

Watermelon is by far, one of the most powerful, body-healing fruits out there! The amazing health benefits of watermelon cover everything from your brain all the way to the cells in your feet. Watermelon is incredibly hydrating (up to 92% water!) and is naturally low-fat. Make this melon a part of your daily diet and you will reap amazing benefits that range from improving cardiovascular health to nourishing your eyes and revving up your immune system! Read below and see for yourself.

**Cardiovascular & Bone Health:** The lycopene in watermelon is especially important for our cardiovascular health and is now being recognized as an important factor in promoting bone health. Consuming large amounts of watermelon has also been correlated with improved

cardiovascular function because it improves blood flow via vasodilation (relaxation of blood pressure). Dietary lycopene (from foods like watermelon or tomatoes) reduces oxidative stress which normally reduces the activity of osteoblasts and osteoclasts (the two major bone cells involved in the pathogenesis of osteoporosis) – this means stronger bones for those consuming lycopene-rich foods. Watermelon is also rich in potassium which helps to retain calcium in your body, resulting in stronger bones and joints.

**Reduces Body Fat:** The citrulline in watermelon has been shown to reduce the accumulation of fat in our fat cells. Citrulline is an amino acid which converts into arginine with help from the kidneys. When our bodies absorb citrulline it can take the step of converting into arginine if so required. Citrulline, when consumed, has the ability to (through a series of steps) block the activity of TNAP (tissue-nonspecific alkaline phosphatase) which makes our fat cells create less fat, and thus helps prevent over-accumulation of body fat.

**Anti-inflammatory & Antioxidant Support:** Watermelon is rich in phenolic compounds like flavonoids, carotenoids, and triterpenoids. The carotenoid lycopene in watermelon is particularly beneficial in reducing inflammation and neutralizing free radicals. The triterpenoid cucurbitacin E is also present in watermelon, which provides anti-inflammatory support by blocking the activity of cyclooxygenase enzymes which normally lead to increased inflammatory support. **Diuretic & Kidney Support:** Watermelon is a natural diuretic which helps increase the flow of urine, but does not strain the kidneys (unlike alcohol and caffeine). Watermelons help the liver process ammonia (waste from protein digestion) which eases the strain on the kidneys while getting rid of excess fluids.

**Muscle & Nerve Support:** Rich in potassium, watermelon is a great natural electrolyte and thus helps regulate the action of nerves and muscles in our body. **Alkaline-forming:** Watermelons have an alkaline-forming effect on the body when fully ripe. Eating lots of alkaline-forming foods (fresh, ripe, fruit and vegetables) can help reduce your risk of developing disease and illness caused by a high-acid diet.

**Improves Eye Health:** Watermelon is a wonderful source of beta-carotene (that rich red hue of watermelon = beta carotene) which is converted in the body to vitamin A. It helps produce the pigments in the retina of the eye and protects against age-related macular degeneration as well as prevents night blindness. Vitamin A also maintains healthy skin, teeth, skeletal and soft tissue, and mucus membranes.

**Immune Support, Wound Healing & Prevents Cell Damage:** The vitamin C content in watermelon is astoundingly high. Vitamin C is great at improving our immune system by maintaining the redox integrity of cells and thereby protecting them from reactive oxygen species (which damages our cells and DNA). The role of vitamin C in healing wounds has also been observed in numerous studies because it is essential to the formation of new connective tissue. The enzymes involved in forming collagen (the main component of wound healing) cannot function without vitamin C.

## MATERIALS AND METHODS

**Drugs and chemicals:** The standard drugs 5-fluorouracil purchased from Local Retail Pharmacy Shop and solvents and other chemicals used for the extraction and phytochemical screening used from Institutional Store and were of AR grade.

**Spectral study:** Primary identification test performed using colour chemical reactions like RP-TLC. Identification of chemical structure of the isolated lycopene was done using UV, IR, NMR and Mass spectroscopy and HPLC.

**Cell culture:** The cell culture human colorectal adenocarcinoma HT-29 cell line was provided by National Centre for Cell Science (NCCS), Pune and was grown in Eagles Minimum Essential Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO<sub>2</sub>, 95% air and the culture medium was changed twice a week.

**Methodology for extraction [6]:** Weigh about 1g of water melon paste into a screwcap vial. Add 50 ml of ethanol and 50 ml of dichloromethane solution cap and shake vigorously or undergo centrifugation. Then collect the supernatant liquid and filter the liquid into a collection flask. Repeat this process 4-5 times, combining the colour liquid in the same collection flask. Transfer the liquid to a separatory funnel and wash with saturated NaCl solution, then with 10% K<sub>2</sub>CO<sub>3</sub> and finally again with NaCl. Save the top organic portion each time, and after the final washing, transfer the organic portion to a beaker and dry it with anhydrous sodium sulphate. Decant the dried organic phase and evaporate the solution to dryness under vacuum to get crude ethanolic extract of watermelon (EEWM).

**Phytochemical screening [7-10]:** Preliminary Phytochemical Screening has to be carried out for the identification of reducing sugars, pentoses, disaccharides, polysaccharides, proteins and amino acids, polyphenols and carotenoids etc.



**Fig-3: EEWM Contain polyphenols**



**Fig-4: EEWM Contain carotenoids**

**Spectral studies:** The IR spectrum of EEWM was recorded by using KBr (FTIR – Bruker, Germany). The  $^1\text{H-NMR}$  spectra of EEWM was recorded on instrument BRUKER NMR spectrometer in  $\text{CDCl}_3$  and  $^{13}\text{C-NMR}$  spectra of EEWM was recorded on instrument JEOL- $^{13}\text{C-NMR}$  spectrometer in  $\text{CDCl}_3$ . The mass spectra of EEWM were recorded by using LC-MS SHIMADZU (Shimadzu Corporation), Mass Spectrometer, QP-1000 Shimadzu, Japan and Negative-ion APCI. The purification and quantification of EEWM was carried out by RF-TLC and HPLC. The IR,  $^1\text{H-NMR}$ , and MASS spectra were used to assign the structure

of bioactive compound lycopene. The spectral data of EEWM have shown the presence of carotenoid lycopene.

**Spectral data of lycopene:** Primary identification test were performed using color chemical reactions like RP-TLC. Identification of chemical structure of the isolated lycopene was done using UV, IR, NMR and Mass spectroscopy and HPLC.

**UV spectra:**  $\lambda_{\max}$  (nm): In methanol 293, 360, 442, 468, 499, %III/II=68.7.  $\lambda_{\max}$  (nm): In hexane 286, 295, 425, 448, 476, 507.

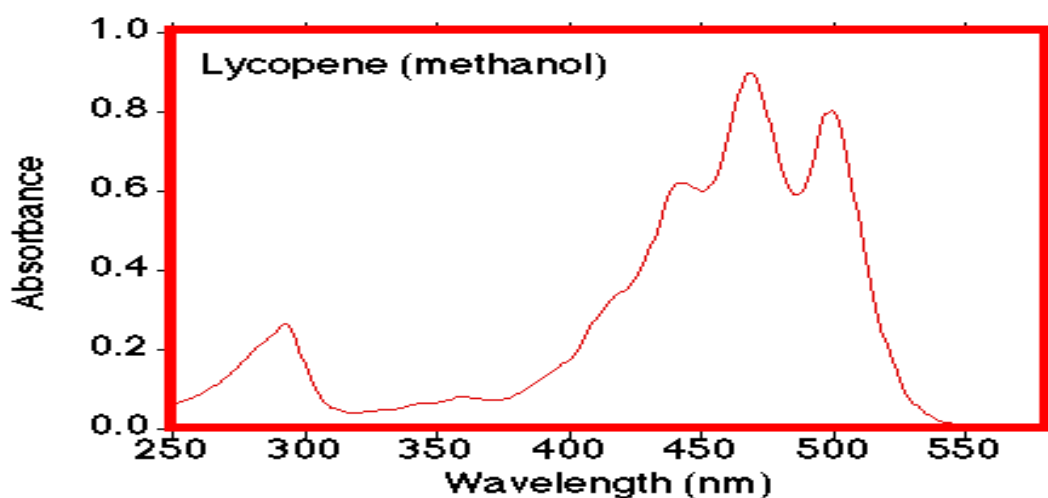


Fig-5A: UV spectra of Lycopene in methanol

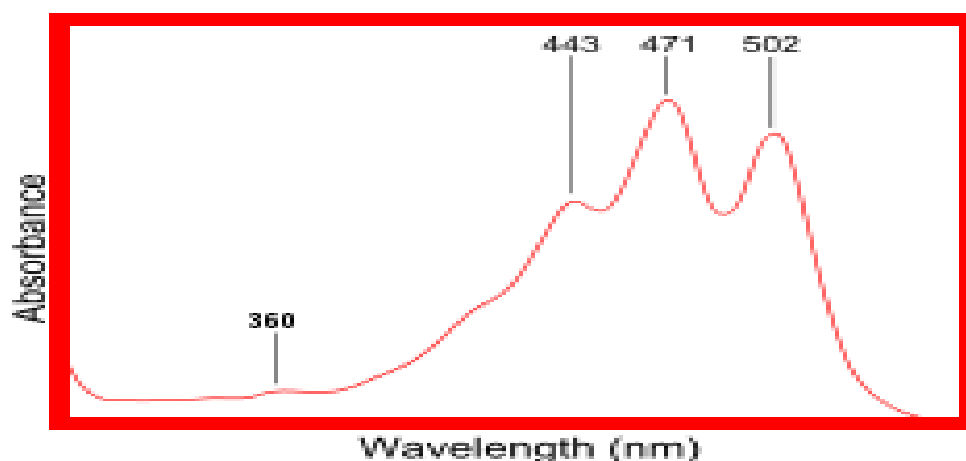


Fig-5B: UV spectra of Lycopene in n-Hexane

**IR spectra:** Vibrational wavelengths of IR Spectrum of extracted lycopene in KBr (FTIR – Bruker) are as follow:

Sl. no	Stretching	Absorption range (cm <sup>-1</sup> )
1.	CH <sub>str</sub> (SP <sup>2</sup> )	3100
2.	CH <sub>str</sub> (SP <sup>3</sup> )	2918.92, 2851.05
3.	C=C <sub>str</sub> (Trans)	1670, 1640
4.	CH <sub>2</sub> (Bending)	1446.92, 1400
5.	CH(Trans OOP)	1101.07, 1000, 957.33
6.	R <sub>2</sub> C=CR	612.84

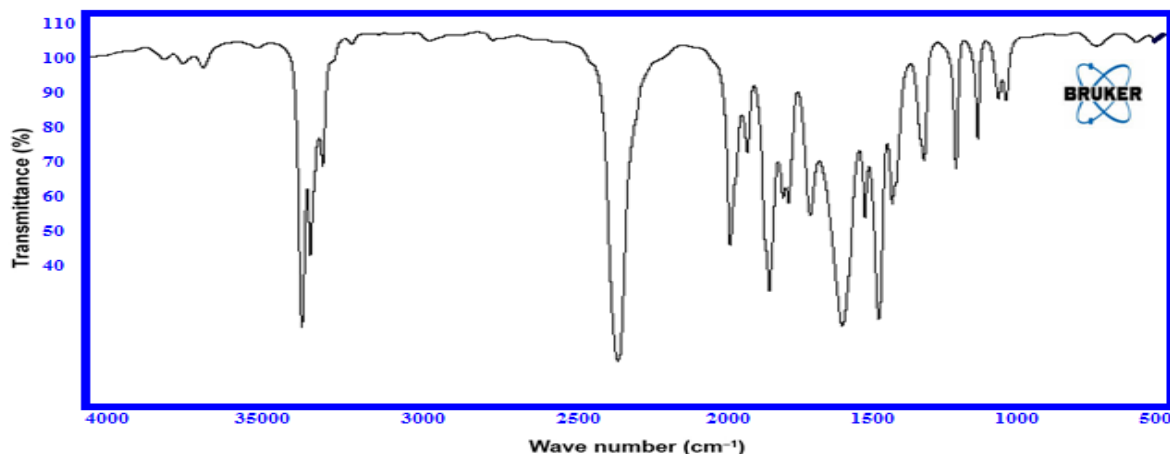


Fig-6: IR spectra of Lycopene

**NMR spectra:** <sup>1</sup>H NMR Spectrum was recorded using <sup>1</sup>H NMR Spectrophotometer, Bruker 300 and 400 MHz Advanced Ultra Shield, and Germany.

**<sup>1</sup>H-NMR d(CDCl<sub>3</sub>):** 5.11 (2, 2'-H), ca. 2.11 (3, 3'-H<sub>2</sub>), ca. 2.11 (4, 4'-H<sub>2</sub>), 5.95 (6, 6'-H), 6.49 (7, 7'-H), 6.25 (8, 8'-H), 6.18 (10, 10'-H), 6.64 (11, 11'-H), 6.35 (12, 12'-H), 6.25 (14, 14'-H), 6.62 (15, 15'-H), 1.688, 1.612 (1, 1'-gem-Me), 1.818 (5, 5'-Me), 1.968 (9, 9', 13, 13'-Me).

**<sup>13</sup>C-NMR d(CDCl<sub>3</sub>):** 131.64 (1, 1'), 124.12 (2, 2'), 26.83 (3, 3'), 40.30 (4, 4'), 139.30 (5, 5'), 125.94 (6, 6'), 124.87 (7, 7'), 135.54 (8, 8'), 136.15 (9, 9'), 131.64 (10, 10'), 125.21 (11, 11'), 137.46 (12, 12'), 136.54 (13, 13'), 132.71 (14, 14'), 130.17 (15, 15'), 25.66, 17.70 (1, 1'-gem-Me), 16.97 (5, 5'-Me), 12.90 (9, 9'-Me), 12.81 (13, 13'-Me).



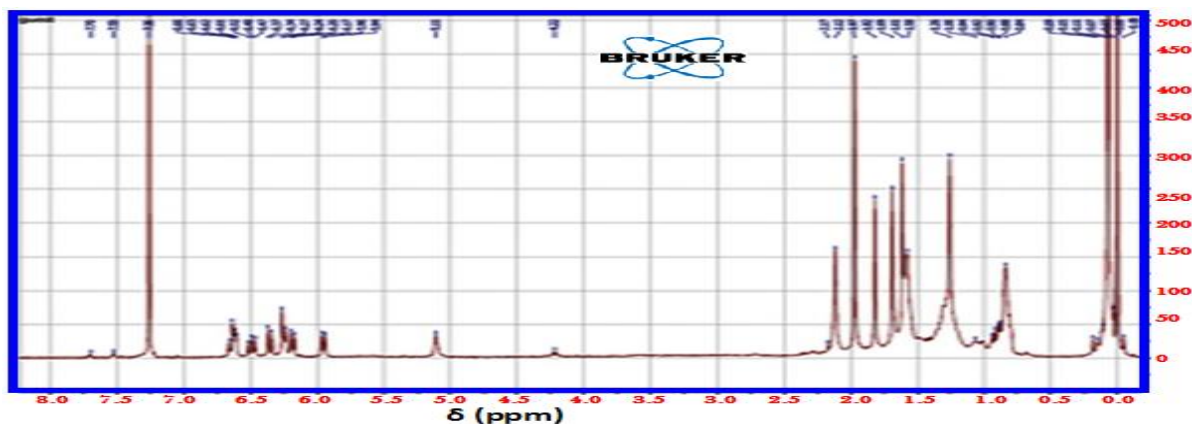


Fig-7A:  $^1\text{H}$ -NMR spectra of Lycopene

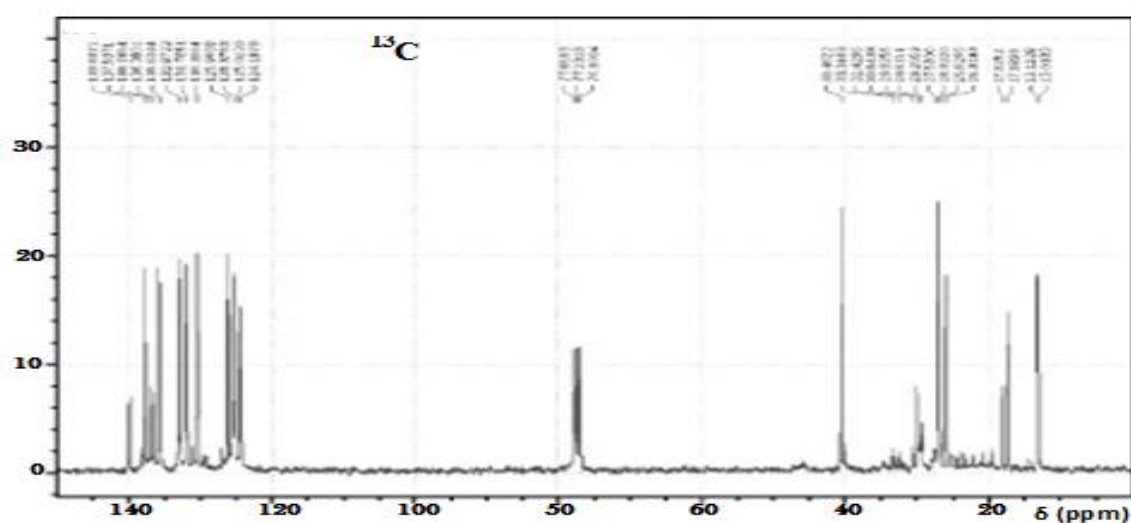


Fig-7B:  $^{13}\text{C}$ -NMR spectra of Lycopene

**Mass spectra:** Negative-ion APCI (Atmospheric pressure ionization) product ion tandem mass spectrum of the lycopene molecular ion radical of  $m/z$  536.43. Collision-induced dissociation was used with argon collision gas at 30 eV in a hybrid quadrupole time-of-flight MS. Note the abundant product ion of  $m/z$  467 corresponding to the elimination of an isoprene group. Because isomeric  $\alpha$ - and  $\beta$ -carotene do not fragment via this pathway, LC-MS-MS was used to detect lycopene selectivity based on selected reaction monitoring of the fragmentation pathway  $m/z$  536 to 467. CID was used to induce fragmentation of the molecular ion of  $m/z$  536. As a result, the fragment ion of  $m/z$  467 was formed by the loss of a terminal isoprene unit. This fragment ion may be used to distinguish lycopene from isomeric  $\alpha$ -carotene and  $\beta$ -carotene that lack terminal isoprene groups. Mol. Wt. 536;  $m/z$ : 536(37%), 145(38%), 119(25%), 105(27%), 93(30%), 91(30%), 81(40%), 69(77%), 41(60%).

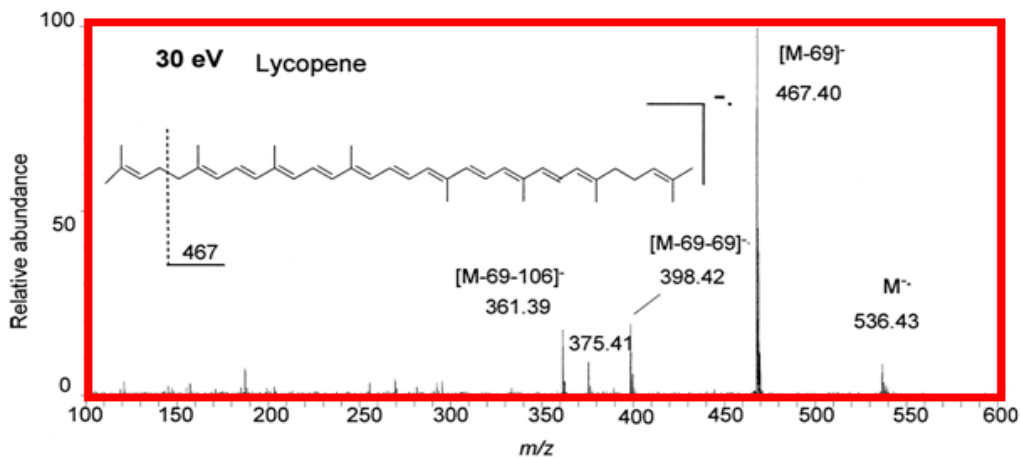


Fig-8A: Mass spectra of Lycopene (APCI)

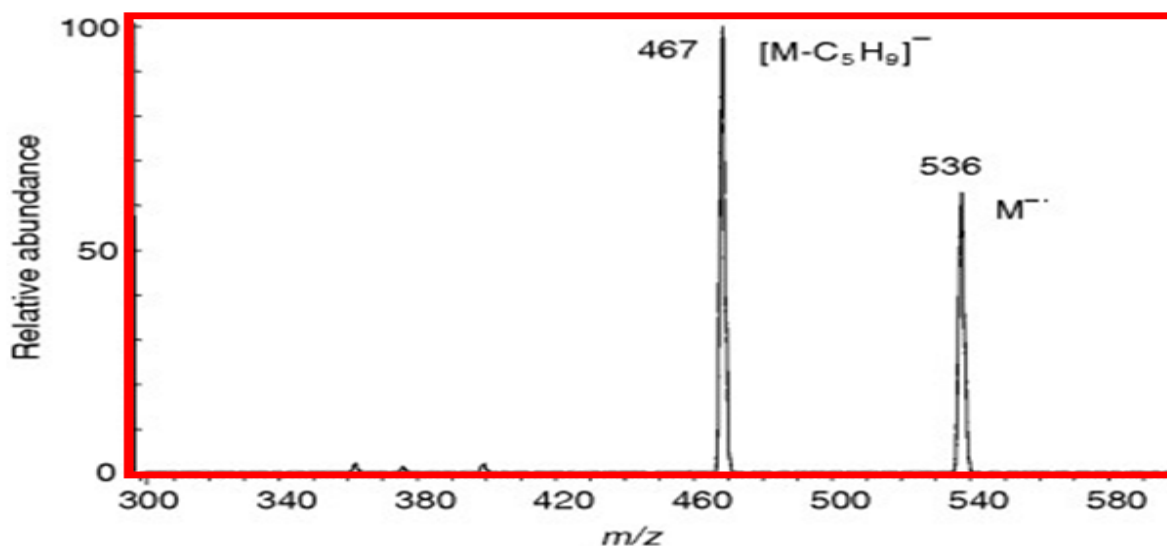


Fig-8B: Negative ion electrospray tandem mass spectrum of lycopene.

**Chromatographic data:** HPLC (column: Nucleosil-300-5 0.4times50 cm, eluent: hexane-N-ethyl-diisopropylamine, 2000:1, flow: 0.6 ml/min, detect : 469 nm) tR = ca. 27 min (all-E isomer).

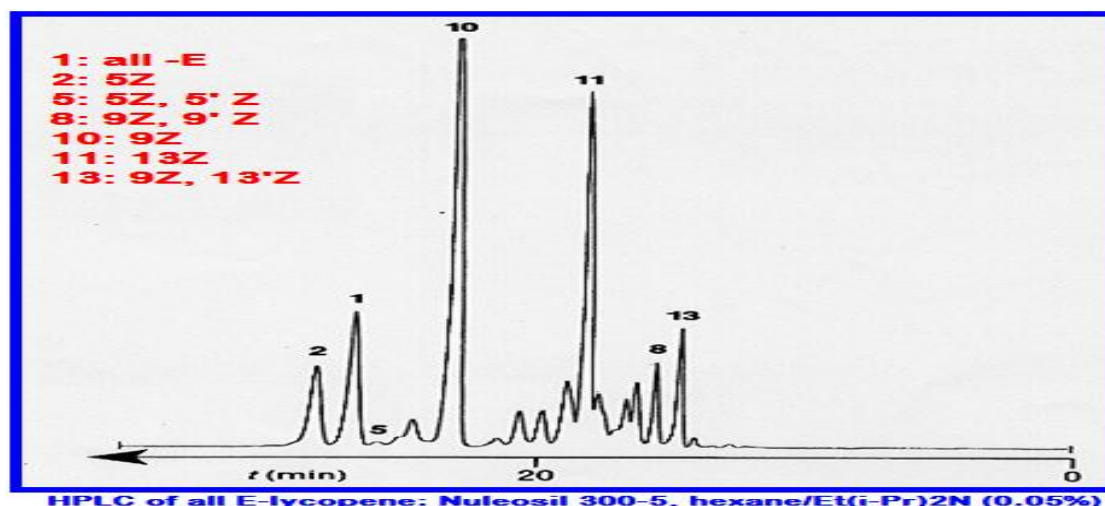


Fig-9: HPLC Chromatogram

### Screening of *in vitro* cytotoxic activity by SRB assay [11, 12]

**Principle:** Sulforhodamine B (SRB) is a bright pink amino xanthine dye with two sulfonic acid group. Under mild acidic conditions, SRB dye binds to basic amino acid residues in trichloroacetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude.

**Procedure:** The monolayer cell culture was trypsinized and the cell count was adjusted to  $0.5-1.0 \times 10^5$  cells/ml using a medium containing 10% new born sheep serum. To each well of the 96 well micro titre plate, 0.1 ml of the diluted cell suspension (approximately) 10,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed once and **10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml** of different concentration of EEWM was added to the cell in a microtitre plate. The plates were incubated at 37<sup>0</sup>c for 72 hrs in 5% CO<sub>2</sub> incubator, microscopic examination was carried out and observations were recorded every 24 hrs. After 72 hrs, 25µl of 50% TCA was added to wells gently such that it forms a thin layer over the test extracts to form overall concentrations 10%. The plates were incubated at 4<sup>0</sup>c for 1 hr. The plates were flicked and washed five times with tap water to remove traces of medium sample and serum and were then air dried. The air dried plates were stained with 100µl SRB and kept for 30 mints at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100µl of 10 mM Tris base was then added to the wells to solubilize the dye[13]. The plates were shaken vigorously for 5 mints. The absorbance was measured using

microplate reader at a 540 nm. The % growth inhibition was calculated by the following formula:

$$\% \text{ cell growth inhibition} = 100 - \left\{ \left( \frac{A_t - A_b}{A_c - A_b} \right) \right\} \times 100$$

A<sub>t</sub> = Absorbance value of test compound

A<sub>b</sub> = Absorbance value of blank

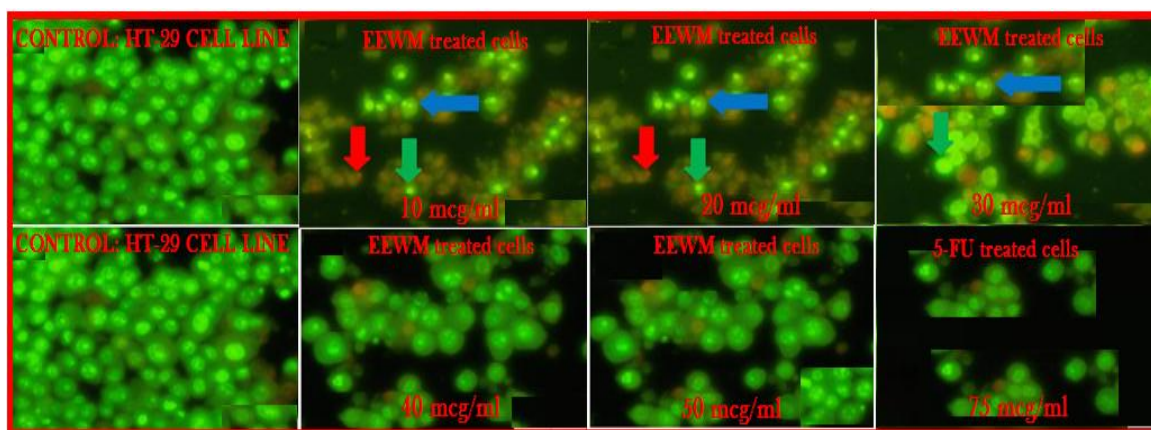
A<sub>c</sub> = Absorbance value of control

## RESULT AND DISCUSSION

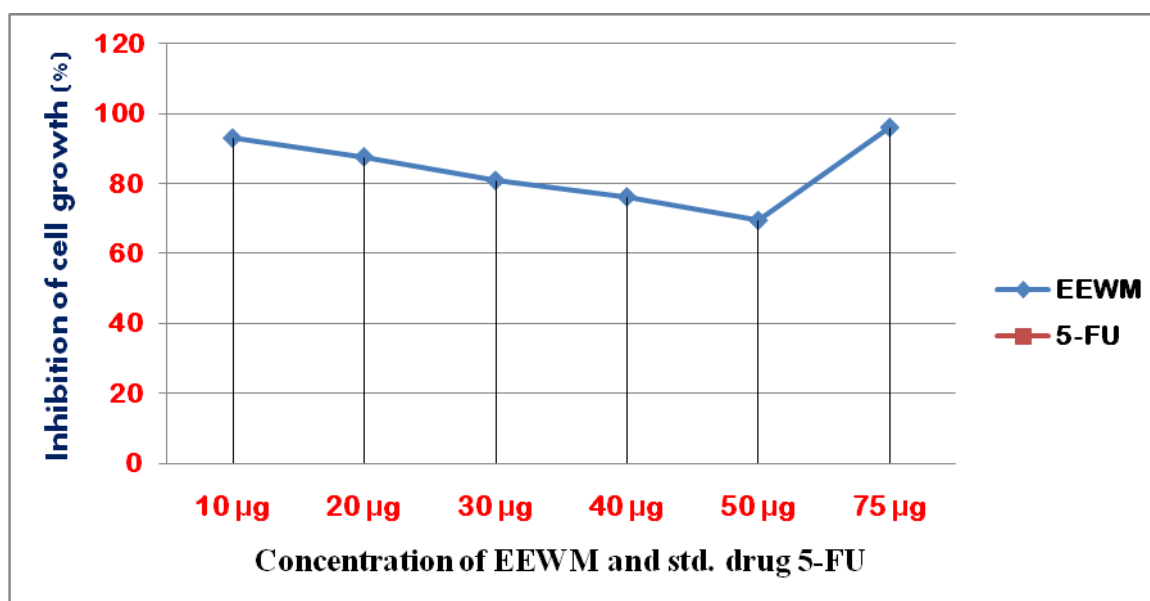
The cell growth inhibition by the extracts such as EEWM against HT 29 cell lines for various concentrations is shown in table 1. As the concentration increase, there is an increase in the cell growth inhibition and it was found that EEWM with the highest **92.95%** growth inhibition at 10 µg (**IC<sub>50</sub> = 2.7 µg/ml**). The IC<sub>50</sub> value of standard drug 5-FU was found to be **1.399 µg/ml** with **95.97%** growth inhibition at concentration 75 µg/ml.

**Table 1: For percentage (%) of cell growth inhibition of EEWM on HT29 Cell lines by SRB Assay**

Serial no.	Concentration of the Extracts	Absorbance of extracts	Inhibition of cell growth (%)
1	10 µg/ml	0.021	92.95
2	20 µg/ml	0.037	87.58
3	30 µg/ml	0.057	80.88
4	40 µg/ml	0.071	76.17
5	50 µg/ml	0.091	69.47
6	75 µg/ml (5-FU)	0.012	95.97
7	Control	0.298	0



**Figure 10:** Acridine Orange / Propidium Iodide of HT-29 cells after treatment at the IC<sub>50</sub> value of EEWM: The red color was produced by PI staining which penetrated the nuclear matter when the cell membrane integrity was disturbed. Cells with an intact membrane are stained green. Apoptotic cells are stained green with nuclei stained red and contain multiple yellow/green dots of condensed nuclei. Necrotic cells were stained bright red due to the influx of PI stain. In the green arrows point to healthy cells, the blue arrows point apoptotic cells with a fragmented nucleus and condensed chromatin, and the red arrow points to a necrotic cell. Magnification = ×100.



**Fig 11: Percentage (%) of cell growth inhibition by EEWM on HT29 cell line.**

## CONCLUSION

The results obtained from the *in-vitro* studies displayed that the ethanolic extracts of the fruit of *Citrullus vulgaris* L. (EEWM) possessed a very good cytotoxic activity against human colorectal adenocarcinoma HT-29 cell line. From the present experimental data it had been concluded that EEWM was exhibiting the potential capability to inhibit the growth of cancer cell when compared with standard drug 5-FU and the cell growth inhibition of EEWM was found to be the highest 92.95% at 10 µg (IC<sub>50</sub> = 2.7 µg/ml). The potential cytotoxic activity of EEWM was mainly due to the present of bioactive compounds lycopene and beta carotene which were characterized by modern analytical techniques such as UV, IR, NMR, Mass spectrometry and HPLC methods.

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