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RESEARCH ARTICLE

EXTRACTION AND DETERMINATION OF LYCOPENE FROM WATERMELON BY DIFFERENT SPECTRAL TECHNIQUES (UV-VIS, FTIR AND GC-MS) FOR IN VITRO ANTIOXIDANT ACTIVITY

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ABSTRACT

Lycopene is a carotenoid found in nature, it is being used for the treatment of various disorders like cardiovascular diseases, osteoporosis, bone health, male infertility, skin protection, and age related muscular degeneration prevention. It exhibits the highest antioxidant activity and singlet oxygen quenching ability of all dietary carotenoid. The red fleshed watermelon juice samples were analysed by UV-VIS spectrophotometer, FTIR spectroscopy and GC-MS. The most predominant volatiles lipid forms of hydrocarbons were identified through GC-MS. The major chemical constituents are identified as N,N'- Ethylenebis [2-[2- hydroxyphenylglycine, Cyclopropanebutanoic acid, n- Hexadecanoic acid, 10-Octadecenoic acid, oleic acid, Heptadecanoic acid, Hexadecanoic acid, 9-octadecenoic acid. The chemical compounds found in groups are Methyl branched fatty acid, ethylester based fatty acid, Glycine based amino acid, and Palmitic acid and oleic acid. The results showed that lycopene content of watermelon juice of approximately 13mg/Kg. The extract of lycopene was subjected to invitro antioxidant activity using three different methods such as DPPH, hydrogen peroxide radical and reducing power assay method. The lycopene exhibited DPPH activity with an ARP value of 0.0035, hydrogen peroxide scavenging activity with IC50 value of 10.636 µg/ml. The extracts had shown significant antioxidant activity against method with IC50 value of 22.70µg/ml.

INTRODUCTION

Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms but not animals. In plants, they contribute to the photosynthetic machinery and protect them against photo- damage. Fruits and vegetables constitute the major sources of carotenoid in human diet (Guddadarangavvanahally *et al*., 2004). They are present as micro-components in fruits and vegetables and are responsible for their yellow, orange and red colors. Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing human diseases including cardiovascular diseases, cancer and other chronic diseases. The antioxidant properties of carotenoids have been the major focus of research. Close to 90% of carotenoid in the diet and human body is represented by beta-carotene, alpha-carotene and lycopene. Different carotenoid patterns were found in red fleshed and yellow fleshed watermelon. Red fleshed watermelon contains high levels of lycopene and varying amount of β carotene (Tadmor *et al*., 2005). Watermelon is under genera of citrullus, which rank among top ten in economic importance among vegetable crops globally (Schaffer and Paris, 2003). Lycopene belongs to the family of carotenoid. It has a structure that consists of a long chain of conjugate double bonds, with two open end rings. The structure lycopene is the longest of all carotenoid. Lycopene (C40H56) molecular weight 536.9 is an unsaturated hydrocarbon carotenoid containing 13 -carbon double bonds 11 of which are conjugated and arranged in a linear array (Agarwal and Rao, 2000; Suja *et al*., 2005 and Sandman, 1994). These conjugated double bonds are responsible for the vibrant red color of lycopene. Lycopene is a lipophilic compound that is insoluble in water but soluble in organic solvents, and it has quenching constant double that of beta carotene and 10 times greater than alpha tocopherol. The quenching ability is directly related to the position of excited state energy levels, which depend on the length of the conjugated carbon double bond chain. As free radicals are electrochemically imbalanced molecules, they are highly aggressive, and are always ready to react with cell components and cause permanent damage. As an antioxidant, lycopene has a singlet oxygen- quenching ability twice as high as that of beta-carotene (vitamin A relative) and ten times higher than that of alpha-tocopherol (vitamin E relative). FTIR spectroscopy is a well established, nondestructive technique for analyzing agricd products. It offers much to the analyst

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because spectral bands may be assigned to specific chemical entities and it provides bands arising from group vibrations with known assignment in most cases. GC-MS is a method used to help identify a mixture of compounds by separating compounds according to each compound's retention time. GC-MS is one of the best techniques to identify the bioactive constituents of long chain branched chain hydrocarbons, alcohols, acids, ester etc. The objective of this study was to develop methodology for the rapid, accurate, and sensitive extraction and determination of lycopene in watermelon using UV-VIS, GC-MS, FTIR spectroscopy and to evaluate the invitro antioxidant activity.

MATERIALS AND METHODS

Plant materials and sample preparation

Red flesh watermelonwere purchased from a local supermarket. The fruits were washed, drained and wiped - dry. It was cut into a few small portions and then blended to paste-like state for approximately 2 minutes using a blender. During the blending process, intermittent stops were required to minimize heating effects on the watermelons. The homogenized sample was centrifuged at 1000g for 30 minutes and at 4^{o}c being filtered under suction. The sample was stored at-20^{o}c until use within a week.

Extraction of Lycopene

Lycopene content was determined according to the method of Davis *et al.* (2007) with some modifications. Approximately 0.6g of sample was weighed and added to 5mL of 0.05% (W/V) BHT in acetone, 5mL of 95% ethanol and 10mL of hexane. The homogenate was centrifuged at 400g for 15 minutes at 4^{o}C. After that, 3mL of distilled water was added. The vials were agitated for 5 minutes and left at room temperature to allow phase separation. The absorbance of upper hexane layer was measured in a 1cm-pathlength quartz cuvette at 503nm using a spectrophotometer. Hexane was used as blank. The lycopene content in the sample was estimated according to the equation.

$$\text{Lycopene (mg/kg tissue)} = \frac{\text{A503 x31.2}}{\text{Mass of tissue (g)}}$$

Where A503 is the absorbance of upper hexane layer Wee Sim Choo and Wai yen Sin (2012). UV-VIS light absorption pattern of lycopene was kinetically monitored in the range of 503nm using ELCO, SL 164 UV- VIS spectrophotometer.

Determination of lycopene by different spectral techniques

Gas chromatography- mass spectrometry

GC-MS technique was used in this study to identify the phytocomponents present in the extract (Markovic *et al*., 2006). An analysis was performed on a JEOL GCMATE II GC-MS with Data system is a high resolution, double focusing instrument, maximum resolution. The electron impact ionisation method was analysed. The linear velocity of the high pure helium carrier gas was 30 cm/s. The injector temperatures were 220 ºC. The oven temperature was programmed from 50 to 250 ºC at 10 ºC/ min and held for 50 min.

Fourier transform infrared spectroscopy

The spectra or fingerprints of the selected samples were obtained using FT-Raman and/or FTIR spectroscopy. The samples of FT-IR were prepared by using potassium bromide disks. FT-Raman spectra were obtained using a PERKIN ELMER SPECTRUM ONE at the resolution 4 cm $^{-1}$ with the spectral range of 4000 – 450 cm-^{1}.

In- vitro antioxidant assay

Determination of free radical scavenging activity

Free radical scavenging activity was determined according to the method of Sies (1996) with some modifications. Lycopene extract (1mL) were prepared and then added to 2mL DPPH solutions (0.05 M) in ethanol, respectively. The reduction of DPPH in the samples was measured at 517nm after 30 minutes against a blank assay (samples added to 2mL of ethanol, respectively).

The percentage of remaining radical was calculated by using this formula:

Percentage inhibition= (Abscontrol- Abssample) /Abscontrol x 100 (Gyamfi *et al*., 1999)

Where, Abscontrol was the absorbance of solution without extract and Abs sample was the absorbance of lycopene extract. The amount of sample required to decrease the initial DPPH concentration by 50%, $EC_{50,}$ was calculated.

The anti-radical power is calculated by using equation:

Anti- radical power (ARP) = 1/ EC_{50}

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20mM) was prepared in phosphate buffer saline (PBS, pH 7.4). One ml of lycopene extracts were added to 2 ml of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 minutes against a blank solution that contained extract or standard in PBS without hydrogen peroxide.

Reducing power assay method

One ml of Lycopene extracts were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.8) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50^{o}C for 20 minutes. To this mixture, 2.5ml of 10% trichloroacetic acids (TCA) was added and then centrifuged at 3000rpm for 10 minutes. The upper layer of the solution (2,5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml, 0.1%) was added and the absorbance was measured at 700nm.

The percentage of reducing power was calculated by using the formula:

Reducing power (%) = Abscontrol- Abssample/Abscontrol

Where, Abscontrol was the absorbance of solution without extract and Abssample was the absorbance of lycopene extracts.

RESULTS AND DISCUSSION

Lycopene red fat soluble pigment found in certain plants and microorganisms. By analyzing the absorption spectra of pure compounds presented in the literature (Gyamfi *et al*., 1999). Solutions of lycopene in n-hexane at different concentrations were prepared and absorbance was measured at 503 nm. It was found that lycopene were the most important contribution in the absorption spectrum bands at 503 nm. The content of lycopene in the samples can be estimated by the molar extinction coefficient. By properly substituting the molar extinction coefficient of lycopene in hexane (17.2 X 10^4 M–1 cm–1) as well as the molecular weight (536.9 g) and by changing the units, the final equation will be

Lycopene content (mg/kg) = A503 X 31.2/g tissue

In this study the red fleshed content of watermelon juice used for extraction and its showed higher yield was approximately 13mg/Kg. The lycopene content of the red fleshed watermelon in this study was higher than those reported by Isabelle *et al.* (2010) (10.95 mg/Kg), and Liana Maria Alda *et al.* (2009) (12 mg/Kg).

This difference was due to red fleshed watermelons varied in their lycopene content depending on genotype and environmental conditions.

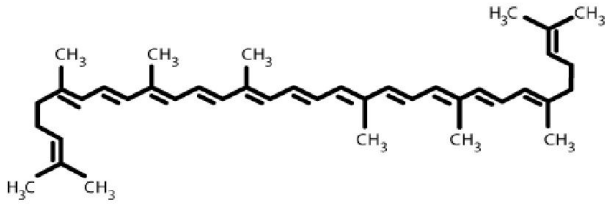

Fig. 1. The structure of lycopene

The lycopene extracts of watermelon has been subjected to GC-MS analysis. Nine chemical constituents have been identified. The major chemical constituents are N, N'- Ethylenebis [2-[2- hydroxyphenylglycine, Cyclopropanebutanoic acid, n- Hexadecanoic acid, 10-Octadecenoic acid, oleic acid, Heptadecanoic acid, Hexadecanoic acid, 9- octadecenoic acid.

The chemical compound in groups was also identified, The Methyl branched fatty acid, ethyl ester based fatty acid, Glycine based amino acid, Palmitic acid and oleic acid were found.

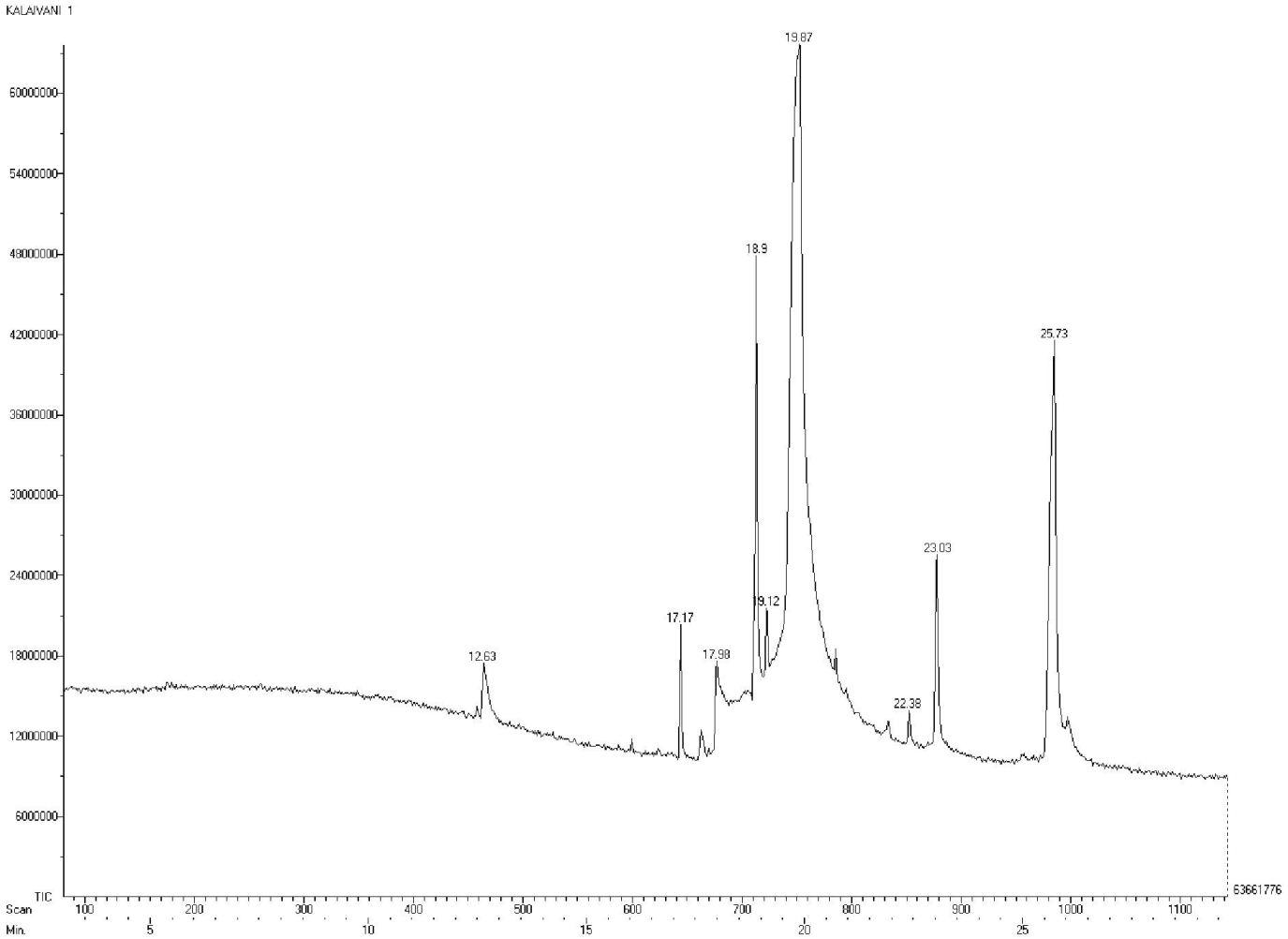

Fig. 2. GCMS spectra of lycopene

GC-MS analysis of lycopene compound

Peak No	Compounds	Retention Time	Molecular Formula	Molecular Weight
1	N, N'-Ethylenebis [2-[2-Hydroxyphenylglycine]	12.78	$C_{18}H_{20}N_2O_6$	360.36
2	Cyclopropanebutanoic acid	17.22	$C_{25}H_{42}O_2$	374.59
3	n- Hexadecanoic acid	17.98	$C_{16}H_{32}O_2$	256.42
4	10- Octadecenoic acid	18.9	$C_{19}H_{36}O_2$	296.48
5	Oleic acid	18.96	$C_{18}H_{34}O_2$	282.46
6	Heptadecanoic acid	19.15	$C_{19}H_{38}O_2$	298.50
7	Oleic acid	20.17	$C_{18}H_{34}O_2$	282.46
8	Hexadecanoic acid	23.07	$C_{35}H_{68}O_5$	568.91
9	9-Octadecenoic acid	25.83	$C_{21}H_{40}O_4$	356.53

Chemical composition of Lycopene

Chemical compound identified in GC-MS	Compound Found in groups
N,N'- Ethylenebis [2-[2- hydroxyl phenylglycine	Glycine based amino acids
Cyclopropanebutanoic acid	Unknown
n- Hexadecanoic acid	Palmitic acid
10.octadecenoic acid Heptadecanoic acid	Methyl branched fatty acids
Hexadecanoic acid 9-Octadecenoic acid	Ethylester branched fatty acids

The chemical compound in groups were also identified, to be the Methyl branched fatty acid, ethyl ester based fatty acid, Glycine based amino acids, palmitic acid and oleic acid was found

The spectral data showed the interpretation of the most significant group frequencies for the functional groups and structural components found in the presents compound. The hydrocarbon and methyl group are molecular fragment that contribute their own set of characteristic of absorptions to the spectrum of the compound. In fact, the bonding between the functional group and the backbone is only the part of the overall picture used for spectral interpretation In this study the changes of chemical compositions and volatile components of lycopene was identified by using Fourier transform infrared spectroscopy technique. Mohie M. Kamil *et al*. (2011) the spectral Peaks of lycopene showed C-H symmetrical stretch alone and C-H asymmetrical stretch methyl at (2960- 2850), C-H stretch of alkane at (3000- 2850), other bands occurs at (1620- 1680) C-H alkene, C-H bends alkene, C-H bends stretch alkene at (900-670) simpler forms of lipids were identified.

In – vitro antioxidant activity

Free radical scavenging activity

DPPH radical was used in the evaluation of free radical scavenging activity of watermelons. 1, 1- Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay is the most widely used methods for screening antioxidant activity. DPPH assay is used to determine the scavenging potential of antioxidant extract based on its capability as hydrogen donator and electron transfer.

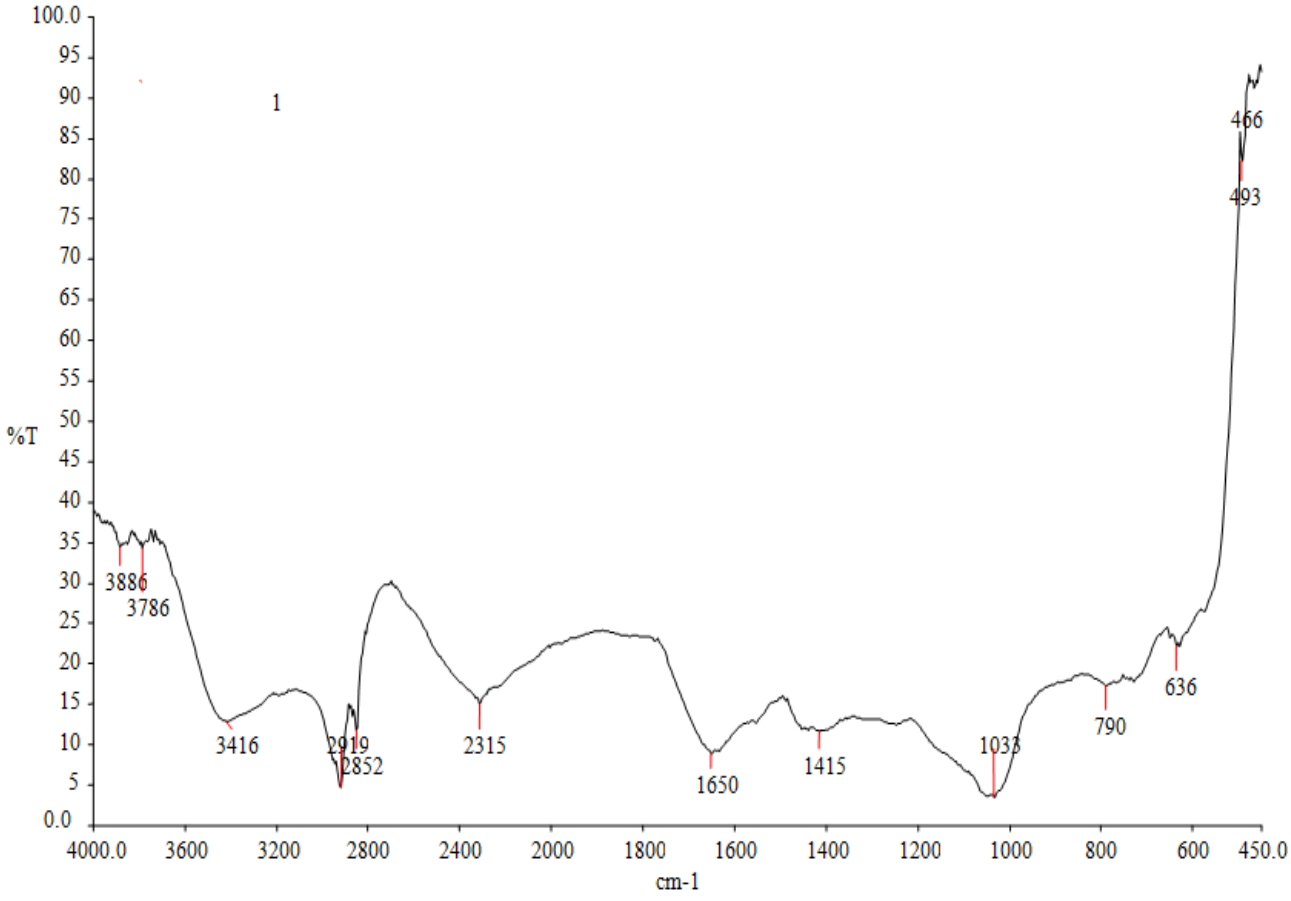

Fig. 3. FTIR Spectrum of Lycopene

The reaction between antioxidant compounds with the stable DPPH radical cause reduction in absorbance. EC50 is defined as the concentration of antioxidant necessary to scavenge 50% of DPPH radicals.

DPPH radical scavenging activity of lycopene

DPPH radical scavenging activity	Activity at 517nm
Test :	3.000
Control :	1.077

The percentage of remaining radical was calculated by dividing the absorbance of the sample with that DPPH control and multiplied by 100. The inhibition of radical was expressed in terms of the EC50 278.55 and ARP values. Higher ARP value of 0.0035 was observed in the lycopene extract, which reflects higher efficiency of antioxidants in the fruit.

Scavenging of hydrogen peroxide

The effect of lycopene extract on hydrogen peroxide radical scavenging activity is shown in table. The lycopene extract showed significant antioxidant activity against hydrogen peroxide radical. The IC50 value of the extract was 10.636 µg/ml.

Hydrogen peroxide radical scavenging activity of lycopene

Hydrogen peroxide radical scavenging activity	Activity at 230nm
Test :	3.000
Control :	3.281

Ferrous ion reducing power assay method

Fe (III) reduction is used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3-} to Fe^{2-} by donating an electron. Amount of Fe^{2-} complex can be monitored at 700nm indicates an increase in reductive ability. The extracts had shown good reducing power. The IC50 value of the extract was 0.227µg/ml.

Ferrous reducing power activity of lycopene

Total reducing power activity	Activity at 700nm
Test :	0.614
Control :	0.265

In this study primary antioxidant property was analysed through in- vitro test using three different methods such as DPPH free radical scavenging activity, hydrogen peroxide scavenging activity and reducing power method with significant IC50 and ARP value respectively. The red fleshed watermelon used for lycopene extraction and its had higher lycopene content and also had higher primary antioxidant activity free radical scavenging, hydrogen peroxide scavenging and reducing power activity.

Conclusion

The presence study revealed that a simple spectral protocol allowed the rapid, accurate, sensitive, and reliable determination of lycopene in watermelon with minimal sample preparation. Expectations should focus on the fields of compond prediction by GC-MS, which will permit higher level analysis with enhanced analytic capabilities and reduced analyzing time. When combined with infrared spectroscopy, the result of a positive identification of qualitative analysis. Therefore, this study had significantly identified the properties of compound present in the lycopene extract, which act as an antioxidant compound.

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