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Lycopene extraction from *Psidium guajava* L. and evaluation of its antioxidant properties using a modified DPPH test

Fabienne Priam [1], Odile Marcelin [1], Roselyne Marcus [1], Louis-Felix Jô. [1], Emilie J. Smith-Ravin [1]

[1](*Université des Antilles, EA929 AIHP-GEODE Groupe BIOSPHERES, 97275 Schœlcher, Martinique)*

Abstract: *The aim of this study was to optimize the extraction and characterization of lycopene, a carotenoid, from a tropical fruit, Psidium guajava L. and to demonstrate its health benefits. Deionised water, mixed with organic solvents, was used for extraction, and the depletion stage was reached in order to define the number of extractions needed to extract most of the lycopene. Optimum extraction was achieved with petroleum ether combined with guava puree:deionised water ratio of 40% using 15 ml of water. Four extractions were a satisfactory compromise to maximize the amount of lycopene recovered and to minimize the use of organic solvent. Excellent purification of lycopene was obtained by HPLC-VIS detection (470 nm) with acetonitrile/methanol/tetrahydrofuran mobile phase. Characterization was confirmed by FTIR analysis. A modification of the DPPH test made it possible to assess the EC_{50}, thereby ranking lycopene among good antioxidants. As Psidium guajava is already widely used in the food industry, these results support claims for health benefits for products derived from guava.*

Keywords - *DPPH, FTIR, HPLC, Liquid-liquid Extraction, Lycopene, Psidium guajava L.*

I. Introduction

A number of tropical fruits are valuable sources of natural antioxidants as they have a high content of carotenoid, polyphenol and vitamin C [1]. Lycopene belongs to the carotenoid family and is a natural pigment that gives the red color to many fruits, including tomatoes, watermelon and guava. It is considered to be an efficient scavenger of free radicals and thus to be an efficient antioxidant [2]. The majority of studies on lycopene have been carried out on lycopene extracted from tomato [3] and there are relatively few studies on lycopene from guava.

In the Caribbean region, pink guava is increasingly used in local processing industries. The fruit can be eaten raw, cooked or processed, and several varieties are produced in Martinique, including Centeno Prolific, Red Supreme Ruby, Beausejour and the Cuban clone Cuba Enana. *Psidium guajava L*. has been shown to release more lycopene than tomato [3] and to be easily absorbed by the body. Pink guava is thus a major source of lycopene [4], and also of vitamin C [5] and polyphenols [6], but little information is available on extraction techniques.

Lycopene is insoluble in water, and solubility and stability are the main problems encountered during the extraction and purification steps. Carotenoids, including lycopene, are generally extracted using conventional methods such as liquid-liquid extraction or solid phase extraction using large amounts of organic solvent, generally followed by a purification step [7]. For this, organic solvents such as hexane [8] and chloroform [9], or solvent mixtures such as methanol-tetrahydrofuran, ethanol-hexane, acetone-ethanol-hexane and ethyl acetate-hexane are often used [10, 11]. The use of supercritical fluid extraction (SFE) has been developed to overcome these drawbacks [9]. Carotenoid extracts are mixed with an oleoresin [12]. However, this method is expensive. As far as purification of lycopene is concerned, high performance liquid chromatography (HPLC) allows a good separation and a reliable identification of carotenoids [13]. Chromatographic methods normally use a C18 or C30 column in isocratic or gradient mode appropriate for many solvents used for the mobile phase. Detection is by diode array or UV/visible spectrophotometry (450-470 nm) [10]. However, lycopene is sensitive to heat, light and oxygen, and great care must be taken as artefacts may be generated by some solvents [14]. Consequently, the development of extraction and separation methods for lycopene and their optimization continue to be of great interest. The aim of this study was to optimize the extraction conditions for lycopene from the *Cuba Enana* guava variety in order to obtain good separation and purification by HPLC. Consequently, we prepared a suitable mixture of extraction solvents and studied the influence of the water content during extraction. The novelty of our method is to have developed a method of extraction and purification which has resulted in a high yield of lycopene of high purity. We also evaluated the antioxidant potential of the resulting lycopene by developing an appropriate test for carotenoids, and more specifically for lycopene, using DPPH (1,1-diphenyl-1- picrylhydrazyl). The antioxidant properties are of particular interest in the context of an increasing consumption of the guava fruit.

II. Materials and Methods

2.1 Assay reagents and standards

Petroleum ether (PE), methanol, tetrahydrofuran (THF) and acetonitrile were obtained from Sigma-Aldrich. Lycopene standard (≥90% purity) was also obtained from Sigma-Aldrich. Ultra-pure water was obtained using a Milli-Q System. For the antioxidant assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) , ascorbic acid, Trolox, α-tocopherol and butylated hydroxyanisol (BHA) were from Sigma-Aldrich.

2.2 Plant material

Cuba Enana guava, one of the sweetest pink varieties, was obtained from the Martinique fruit orchard "Association Vergers et Jardins Tropicaux (AVJT)". One hundred and seventy five pink guavas were harvested at the transition from mature to ripe stage (turning stage) and divided into five batches. The fruits were then cut into quarters, frozen in liquid nitrogen and stored at -80°C until use. For extraction, the fruits were thawed the day before at +4°C, weighed and crushed in a Kenwood mill for 5 min. at room temperature. The resulting puree was sieved on a 2 mm sieve and aliquots were stored at -20°C.

2.3 Extraction

2.3.1 Ratio test

To determine optimum extraction conditions, several guava puree/(guava puree + deionised water) ratios were tested using guava puree mixed with either 15 ml or 10 ml of deionised water, corresponding to ratios ranging from 71% to 25% (w/w). First, 15 ml of deionised water were mixed with varying quantities of guava puree (g): 37.5 g (ratio 71%), 30 g (ratio 67%), 20 g (ratio 57%), 15 g (ratio 50%), 10 g (ratio 40%), 7.5 g (ratio 33%) and 5 g (ratio 25%). Similarly, 10 ml of deionised water were mixed with varying quantities of guava puree (g): 25 g (ratio 71%), 20 g (ratio 67%), 13.5 g (ratio 57%), 10 g (ratio 50%), 6.5 g (ratio 40%), 4.88 g (ratio 33%) and 3.25 g (ratio 25%).

Next, each mixture was supplemented with 50 ml of PE and homogenised in an Ultra-Thurax (IKA Janke and Kunkel T25 Labortechnik) for 5 minutes (power setting 7-8). The mixture was centrifuged for 10 minutes at 10,000 rpm at 20°C. The yellow-orange supernatant, corresponding to the carotenoid-polyphenol extract, was recovered. To remove the polyphenol phase, liquid-liquid extraction was performed using 50% methanol and water, as carotenoids are insoluble in methanol [15]. The carotenoid phase was then recovered for each ratio, and OD readings were made by spectrophotometry at 470 nm. Samples were evaporated with nitrogen at room temperature, and resuspended in THF for purification and analysis by HPLC at 470 nm. Unused samples were evaporated and then stored at -80°C. At all stages of the extraction, the flasks were covered with aluminium foil and protected from light to prevent potential degradation of the carotenoids.

2.3.2. Depletion analysis

A depletion test was performed using the best ratio obtained in the above ratio test. Extraction on the same sample was repeated ten times in order to determine the optimum number of extractions for achieving complete depletion and the lycopene concentration was determined by spectrophotometry at 470 nm.

2.4 Spectrophotometry and HPLC conditions

The UV-VIS spectrum of lycopene and the lycopene concentration were determined by spectrophotometry at room temperature using a quartz cuvette with a 1 cm path length and a double beam UV-VIS absorption spectrophotometer (VARIAN CARY 50 UV-VIS) using a molar extinction coefficient of 1.585 x 10^5 $M^{-1}cm^{-1}$. Dilute extracts were analyzed at a wavelength of 300-600 nm using THF as blank and in triplicate. After extraction, lycopene was purified by HPLC using a Varian ProStar 325 UV detector. For this purpose, the extract stored at -80°C was, after thawing, resuspended in 1 ml THF [16]. A 20 µl loop was used for manual injection into a preparative reverse phase C18-type 5 µm, 150 x 10 mm column (RESTEK). The mobile phase was a ternary solvent mixture consisting of methanol-THF-acetonitrile (55:35:10 v/v/v), with isocratic elution for 10 min at a flow rate of 3 $ml.min^{-1}$ and a column temperature of 30°C regulated by a thermostat (CROCO-CIL). Lycopene was recovered after each injection in an opaque vial, evaporated under a nitrogen stream and stored at -80°C. Detection and quantification were performed using a UV-VIS detector at 470 nm. The lycopene concentration was determined in triplicate by UV-VIS spectrophotometry using the Beer-Lambert law with a molar extinction coefficient of 1.585 x 10^5 $M^{-1}cm^{-1}$ at 470 nm.

2.5 Fourier Transform IR (FTIR) spectroscopy

Infrared spectrophotometry was used to identify and characterize the lycopene molecule in the samples. The FTIR spectrometer used was a Bruker ALPHA Fourier Transform spectrometer in transmittance with KBr pellets. Pure lycopene powder was mixed with KBr powder and pressure was applied to make the KBr/lycopene pellets for measurement of the IR spectrum with a spectral resolution of 4 cm^{-1}, using 16 scans in the 400 cm^{-1} to 4000 cm^{-1} range.

2.6 Antioxidant assay

Anti-free radical activity was determined using the free radical DPPH [17]. In its free radical form, DPPH absorbs at 517 nm, but its absorbance decreases upon reduction by an antioxidant. The free radical scavenging activity of the extracted lycopene was evaluated by its reactivity in DPPH assays from the change in absorbance at 517 nm using the method developed by [18, 19] with modifications. Ascorbic acid, Trolox, α-Tocopherol and BHA were used as antioxidant standards. Due to the fact that lycopene is insoluble in pure ethanol, the solutions of DPPH (10^{-3} M), lycopene and standards were appropriately diluted with an ethanol-THF (1:2, v/v) mixture. A dilution series was prepared in the following concentrations: C_0 = 250 mg.L^{-1}, C_1 = 187.5 mg.L^{-1}, C_2 = 125.6 mg.L^{-1}, C_3 = 75.3 mg.L^{-1}, C_4 = 50.5 mg.L^{-1}, C_5 = 25.2 mg.L^{-1}, C_6 = 12.6 mg.L^{-1} and C_7 = 5.05 mg.L^{-1}. One ml of DPPH solution was added to the assays and solvent was added to the controls. After 30 min. incubation, the absorbance at 517 nm was read on a Jenway 6715 UV10 spectrophotometer. The free radical scavenging activity was determined by measuring the percentage inhibition of the absorbance of DPPH by antioxidants. The EC_{50}, which is the concentration of antioxidant required to reduce the initial DPPH concentration by 50%, was therefore calculated for each sample.

III. Results and Discussion

3.1 Extraction

To limit the use of several organic solvents, petroleum ether was chosen for optimum extraction of lycopene [20]. Centrifugation resulted in the separation of four fractions, F1, F2, F3 and F4 from the top to the bottom of the vials (Fig. 1A). Fraction F1was the supernatant containing polyphenols and carotenoids. Fraction F2 was made up of guava pulp. Fraction F3 was aqueous. Fraction F4 was a pellet containing the guava parenchymal cell walls and stone cell walls.

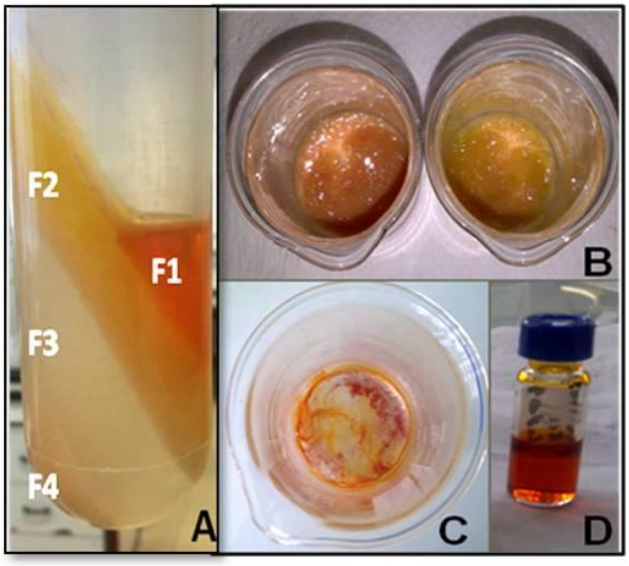

Figure 1. Extraction of carotenoids in petroleum ether. (A) Separation into various fractions (F1, F2, F3 & F4) after centrifugation at 10,000 rpm at 20°C for 10 minutes. (B) Bleached appearance of the guava puree after three extractions. (C) Evaporation of the extract to obtain an orange-red powder. (D) Resuspension of the powder in 1 ml of THF for HPLC

In order to extract the maximum quantity of lycopene, various guava puree/(guava puree + deionised water) ratios were tested with either 10 ml or 15 mL of purified water (Fig. 2). The optimum ratio obtained was 40% with 15 or 10 mL water. With 10 mL of water, the lycopene concentration obtained by spectrophotometry at 470nm was 2.11 mg of lycopene/100 g of guava puree (ratio 40%) whereas with 15 mL of water, 3.26 mg of lycopene/100 g of guava puree were extracted for the same ratio (40%). An adequate amount of water may act as an interface in the guava puree. Its polar protic power is thought to allow the extraction of lycopene from the cell, which subsequently migrates to a non-polar aprotic medium (petroleum ether) having a higher affinity for the compound.

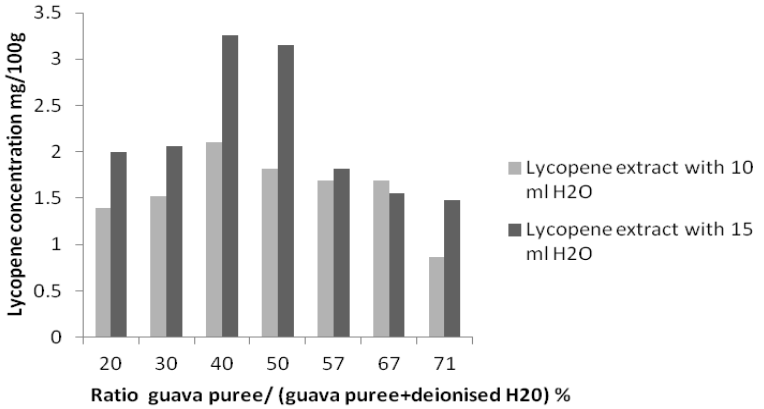

Figure 2. Determination of the best guava puree/(guava puree + deionised water) ratio with 10 mL or 15 mL deionised water

Depletion analysis showed that extracting the optimum amount of carotenoids required only four extractions (Fig. 3), thereby avoiding the excessive use of organic solvents. Examination of the color of the guava puree before and after the four extractions showed a bleached appearance which corresponds to the depleted sample (Fig.1B).

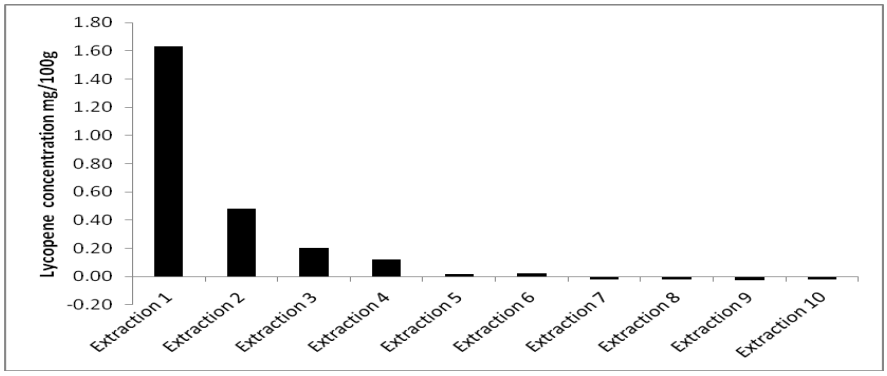

Figure 3. Depletion analysis using a guava puree/(guava puree + deionised water) ratio of 40% with 15 ml deionised water.

The final separation of carotenoids from polyphenols was performed by liquid-liquid extraction with methanol and water. Indeed, water caused a demixing phenomenon between petroleum ether and methanol, causing the polyphenols to migrate to the aqueous-alcoholic phase, a phenomenon which has not been previously reported in the literature. The carotenoid extract was then evaporated under nitrogen and an orange-red powder was obtained (Fig. 1C). For the HPLC purification, the powder obtained was resuspended in 1 ml of THF (Fig. 1D).

3.2 Spectrophotometry and HPLC

The spectral features of lycopene/carotenoids were visible in the spectrum of the extract obtained, diluted 1:10 in petroleum ether (PE). Three characteristic lycopene peaks were identified by spectrophotometry with scan wavelengths of 300 to 600 nm (Fig. 4A). The first peak is at 450 nm, the second at 470 nm and the third at 503 nm, the maximum peak being at 470 nm. The peaks were consistent with the spectra described in the literature at 450, 470 and 503 nm [21]. Fig. 4b represents the chromatogram of lycopene extracted by our method and Fig. 4c represents the chromatogram of a commercially available lycopene standard. Analysis showed that there was similarity between the two chromatograms. The retention time of the extracted lycopene was 11 minutes. The surface areas of the peaks and the absorbance values were similar in the three replicates, confirming the repeatability of the method and the fact that the HPLC conditions were appropriate for optimum purification. The mean of the three peak areas corresponded to a concentration of 4.23 ± 0.20 mg lycopene/100 g of guava puree. The concentration of lycopene obtained by HPLC was consistent with that obtained by spectrophotometric assay. The average concentration was 3.26 mg lycopene/100 g of guava puree. This value is in agreement with the results of studies carried out by [22] who found a concentration of 3.78 mg lycopene/100 g in Malaysian guava. The chromatograms and spectra confirmed the advantage of using the ternary solvent (acetonitrile-methanol-THF) under isocratic conditions in a preparative column to prevent excessive increase in

pressure, thereby ensuring good separation of carotenoids. In addition, the chromatogram shows that the extract was virtually pure, confirming that the extraction conditions were optimum. The purity of the purified lycopene (>95%) appeared to be better than that of the lycopene standard (≥90% purity). Indeed, the chromatogram of the lycopene standard shows a shoulder peak before the lycopene peak compared with our purified lycopene. It is therefore evident from Fig. 4B and Fig. 4C that the lycopene extracted by our method is of greater purity than the commercially available lycopene.

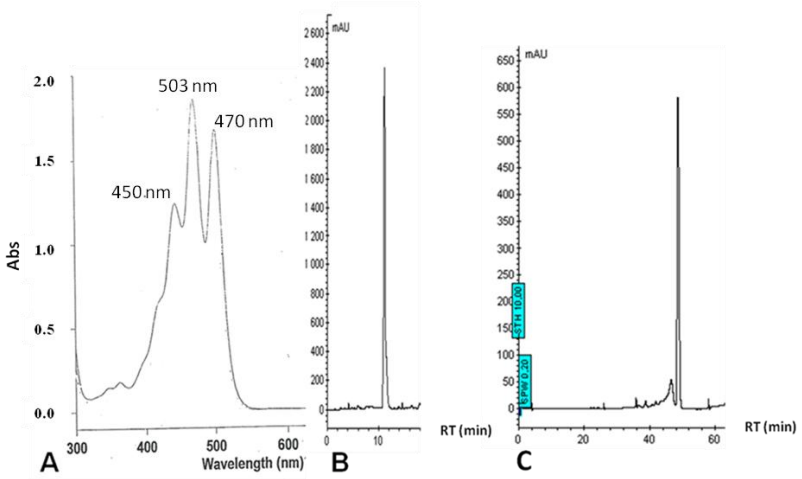

Figure 4. Spectrum and HPLC chromatograms of lycopene. (A) Spectrum of carotenoid phase obtained by UV-VIS spectrophotometry. (B) HPLC chromatogram of lycopene extract. (C) Peak for lycopene standard.

3.3 FTIR characterization

The spectrum of the purified lycopene obtained by FTIR spectroscopy (Fig. 5A and Fig. 5B) is in agreement with published data [23]. The main wavelengths of the vibration wave FTIR spectrum of lycopene were as follows:

λ=2918 cm^{-1}: CH_{str} (sp^2)
λ=2851 cm^{-1}: CH_{str} (sp^3)
λ=1744 cm^{-1}: C=C_{str} (Trans)
λ=1448 cm^{-1}: CH_2 (Bending)
λ=1166.958 cm^{-1}: CH (Trans OOP)

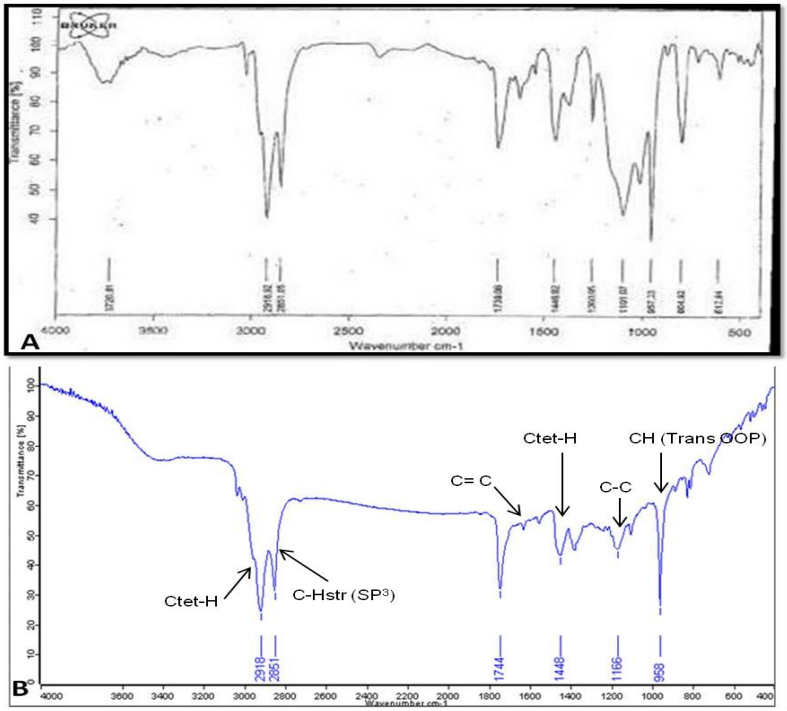

Figure 5. FTIR spectra. (A) Spectrum of lycopene standard. (B) Spectrum of purified lycopene.

3.4 Antioxidant activity

The dose-response curve shows the scavenging effect on free radicals of the lycopene standards and the purified lycopene (Fig. 6). The concentrations of the standards and of the lycopene sample required to reduce the absorbance of DPPH by 50% were as follows: 18.3 $mg.L^{-1}$, 27.3 $mg.L^{-1}$, 35.3 $mg.L^{-1}$, 99.1 $mg.L^{-1}$ and 130.2 $mg.L^{-1}$ for Trolox, vitamin C, α-tocopherol, BHA and lycopene, respectively. This assay required the use of a suitable solvent (ethanol-THF 1:2 v/v) to allow the carotenoids, DPPH and standards to be resuspended under the same conditions. Vitamin C showed a strong antioxidant activity, while the antioxidant effect was observed more rapidly with Trolox. The antioxidant activity of the purified lycopene was close to the BHA dose required for 50% scavenging of DPPH radicals. The profiles of the curves for ascorbic acid and α-tocopherol, which measure their antioxidant activity, were similar to the curves obtained by [18].

The DPPH test is a rapid method for determining antioxidant activity. However, DPPH only dissolves in organic solvents, including methanol and ethanol. This makes it difficult to evaluate the antioxidant properties of lycopene as carotenoids are scarcely soluble in ethanol or methanol [24]. For this reason, as mentioned above in Materials and Methods, it was necessary to dilute the solutions of DPPH, lycopene and standards in an ethanol-THF (1:2, v/v) mixture.

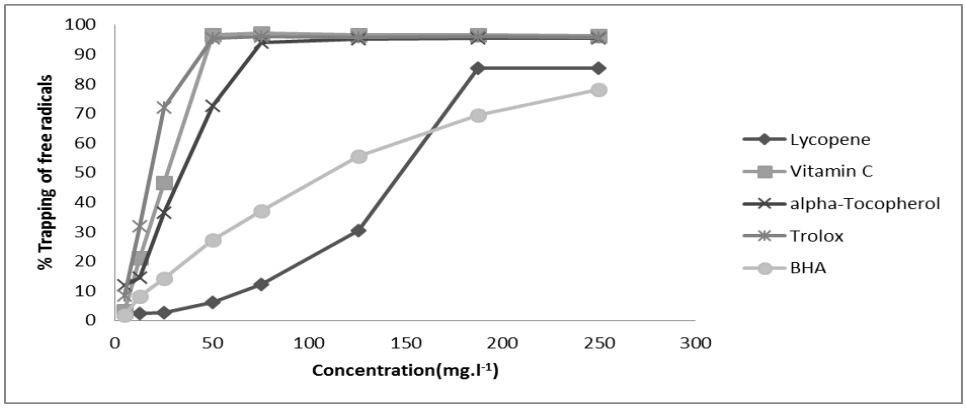

Figure 6. Antioxidant activity of lycopene and various standards: Vitamin C, Trolox, α-Tocopherol and BHA

IV. Conclusion

The main aim of this study was to develop an improved technique for the extraction and purification of lycopene, and to demonstrate its antioxidant property. First, our results show that, under our experimental conditions, four extractions were necessary in order to recover the maximum quantity of lycopene using depletion analysis. Secondly, we optimized the extraction method for lycopene by varying the amount of water in the puree/(puree + deionized water) ratio in order to obtain a high yield. The best ratio obtained was 40% with 15 mL water and 10 g of guava puree. The purification conditions for lycopene were improved using HPLC on a preparative reverse phase C18-type column and isocratic elution *(*methanol-THF-acetonitrile 55:35:10 v/v/v). This method gives a purified lycopene sample with better purity than the commercially available lycopene standard. Furthermore, the antioxidant activity of the lycopene was demonstrated using a modified DPPH test. The antioxidant activity suggests health benefits in addition to the nutritional benefits of products derived from the tropical pink guava.

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