



Carotenoid Analyses and Antibacterial Assay of Annatto (*Bixa orellana* L.), Carrot (*Daucus carota* L.), Corn (*Zea mays* L.) and Tomato (*Solanum lycopersicum* L.) Extracts

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Abstract

Carotenoids are secondary metabolites included in the class of tetraterpenoids commonly found in fruits and vegetables. The study is conducted to analyze the carotenoid content of annatto, carrot, corn, and tomato. Specifically, it aimed to determine the amount of total carotenoids in each plant sample and to separate the components qualitatively using thin layer chromatography (TLC). In addition, the study compared the effectiveness of the plant sample extracts against gram positive and gram negative bacteria and related the total carotenoid content to the antibacterial activity. The total carotenoid (TC) content of the plant samples was measured using the method of Tao et al. with some modifications. Results showed that annatto, carrot, corn, and tomato extracts have 931.30, 102.90, 123.90, and 192.23 $\mu\text{g/g}$ of total carotenoids, respectively. Thin layer chromatographic analysis identified two similar spots for all plant samples with retention factor (Rf) values of 0.00 and 0.98. Annatto, carrot and tomato extracts exhibited antibacterial property against *Staphylococcus aureus*. The mean zone of inhibition of annatto extract was 9.17 mm and was found to be significantly highest among the treatments but lower than the positive control, Streptomycin, with 34.67 mm. The extracts did not inhibit the growth of *Escherichia coli*. Annatto extract which has the highest total carotenoid content also exhibited the highest mean zone of inhibition for *S. aureus*. Thus, the use of annatto extract can inhibit food spoilage caused by *S. aureus*.

Keywords: Carotenoids, anti-bacterial activity, thin layer chromatography, gram positive, gram negative, total carotenoid content, retention factor, plant extracts.

Introduction

Nowadays, different plant and artificial sources of food additives especially colorants have been used in industry¹. However, consumers' demand for synthetic colorants has been decreasing through the years. Food safety and health hazard like cancer were attributed to the use of synthetic colorants². Strict government regulations prompted the industry to shift to natural colorants like that of found in *Monascus sanguineus*³ and *Punica granatum*⁴. One of the most important natural food colorants are carotenoids.

Carotenoids are secondary metabolites included in the class of tetraterpenoids commonly found in fruits and vegetables. There are over 600 known carotenoids including lutein, cryptoxanthin among others. Some of these organic compounds are known to be precursors of the essential nutrient, Vitamin A.

The health benefits of carotenoids same with phytochemicals, tannins⁵ and polyphenols⁶, have been well documented by many researchers. Lycopene, a carotenoid from tomato, has antioxidant activity. It is known to induce cell to cell communication, modulates hormones and improves immune

system⁷. Also, Kritchevsky et al. suggested that carotenoids can be effective inhibitor of heart disease and risk-reducer to some types of cancer⁸. Xanthophylls, lutein, and zeaxanthin have been associated with improving eye health⁹. Aside from their health benefits, carotenoids play important role as food ingredients due to their provitamin activity. Moreover, recent studies have shown that carotenoids have potential antimicrobial activity¹⁰.

The current data on food carotenoids are still questionable¹¹. Carotenoids vary qualitatively and quantitatively from food to food and within each species. Tomato is known to have lycopene; carrot and corn have carotenes, and annatto is known to contain a unique carotenoid, bixin. The need for analysis of carotenoids and inclusion of this information on food composition data bases are important since carotenoid content varies largely with maturity, variety, soil, light intensity among others¹². Also, there are carotenoid losses during postharvest storage¹¹.

Carotenoids are of great importance in food industry since it can serve as colorants which can replace synthetic ones believed to cause cancer. Their possible antimicrobial activities can help lessen food spoilage. Therefore, it is imperative to quantify and

qualify the carotenoids of different plant samples and determine their antibacterial property in preventing spoilage when used in the food industry, hence, this study.

Material and Methods

Air-dried and pulverized samples namely annatto seeds, carrot taproots, corn kernels and tomato fruits were collected and prepared at Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. Each sample was placed in a polyethylene plastic bag and sealed. These were stored in a refrigerator without light until use.

Total Carotenoid Analysis: The methodology for carotenoid analysis was adapted from Tao *et al.* (2010) with some modification and optimization. The samples were air dried for three days and oven-dried at 40°C for 2 hours. About 500 mg of the plant powder was transferred in a 50 mL centrifuge tube and extracted three times with 10 mL ethanol each using vortex mixer for 1 min. The mixture was stood up until particles settled. Supernatants were collected and measured. The final volume of the carotenoids extract was adjusted to 75 mL by adding 95 % ethanol. The absorbance value of the carotenoids extract was determined by Ultraviolet – Visible (UV-Vis) spectrophotometer at 450 nm. These steps were done for all plants samples with three replicates each¹⁰.

The total carotenoid yield ($\mu\text{g/g}$ dried weight) was calculated according to the formula as

$$\text{Total carotenoid yield } (\mu\text{g/g dried weight}) = \frac{V(A - 0.0051)}{0.175W}$$

where A – absorbance value of diluted extraction at 450 nm; V – final volume of the extract (mL); 0.175 – extinction coefficient of carotenoids; and W – weight of dried powder (grams).

Thin Layer Chromatographic Analysis: The plant samples were extracted with absolute ethanol for three days. The extracts were concentrated using rotary evaporator. On a thin layer chromatography (TLC) plate, a baseline was drawn using pencil at the bottom and top approximately 1 cm from the ends. Sample extracts were applied on the TLC plate using a capillary tube. A beaker with solvent and watch glass as lid served as the development chamber. The development chamber was lined inside with a filter paper and poured with the solvent mixture to allow saturation with the solvent. The solvent system used was 5% methanol in xylene instead of 5% methanol in toluene as suggested by Rodriguez-Amaya¹¹. Additional volume of the solvent mixture was poured into the chamber but below the baseline of the TLC plates.

The TLC plate with spotted extracts was transferred to the development chamber taking care that the baseline was above the solvent system. The distance traveled by the spot and solvent were noted. To visualize the spots on the TLC plates,

ultraviolet lamp and iodine chamber were used. The spots were marked using a pencil. Retention factor (*R_f*) was computed using the formula

$$R_f = \frac{\text{distance traveled by the spot}}{\text{distance traveled the solvent}}$$

The TLC analysis was done three times to replicate the spots that were produced.

Antibacterial Assay: This test was done at the Center for Tropical Mushroom Research and Development (CTMRD), Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. Disc diffusion method was employed to determine the antibacterial activity of each plant extract.

Preparation of test organisms: Pure cultures of *Staphylococcus aureus* and *Escherichia coli* were obtained from the culture collection of CTMRD. The cultures were inoculated in test tubes with nutrient broth and incubated at 37°C for 24 hours. The turbidity of the bacterial suspension was compared with 0.5 McFarland standard with approximately 1.5×10^8 bacteria/mL.

Preparation of treatment disc and nutrient agar: A newly prepared filter disc measuring 6 mm diameter was placed in Petri plates and sterilized in an autoclave at 15 pound per square inches (psi) for 20 minutes. The sterilized disc was loaded with approximately 1 mL of the plant extracts and air-dried for several minutes. Ethanol and streptomycin were also loaded on the disc which served as the negative and positive control, respectively.

Thirty nine grams (39 g) of nutrient agar were boiled in 1 L distilled water and dispensed into flask covered with cotton and secured with aluminum foil. The prepared medium was sterilized at 15 psi for 20 min using an autoclave.

In-Vitro bioassay test: Approximately 15-20 mL of nutrient agar with temperature of 40-45°C was aseptically poured onto the sterilized plates. The sterilized cotton swab previously dipped in the bacterial suspension was swabbed on the surface of the nutrient agar plates for three times to facilitate even distribution. The air-dried discs loaded with extracts were seeded equidistantly using flame sterile forceps. Different forceps were used in seeding each treatment disc. Plates were inverted and incubated at 37°C for 18-24 hours. Zone of inhibition was measured to the nearest millimeter using a vernier caliper after incubation. Appearance of zones of inhibition means that the extract is effective.

Statistical Analysis: Data for total carotenoid analysis and antibacterial assay were analyzed using analysis of variance in one way classification analysis. Duncan's Multiple Range Test (DMRT) was used to compare treatments and control means at 5% level of significance. The Statistical Program for Social Science (SPSS) software was used for the statistical analysis.

Results and Discussion

Total Carotenoids of the Plant Samples: The total carotenoid (TC) content of tomato, annatto seeds, corn, and carrots is presented in table 1. The carotenoid content of the samples ranged from 102.90 to 931.30 µg/g.

From these values, annatto has the largest TC content while carrot has the lowest among the four samples. This suggests that annatto seeds can be a great source of carotenoids.

Table-1
Total carotenoid content of annatto, carrot, corn, and tomato

Plant samples	Total carotenoid content (µg/g)
Annatto	931.30 ^a
Carrot	102.90 ^c
Corn	123.90 ^c
Tomato	192.23 ^b

Note: Means having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance.

Analysis of Variance (ANOVA) revealed that there are significant differences among the treatments in terms of their total carotenoid content at 5% level of significance. Duncan's Multiple Range Test (DMRT) shows that annatto has significant amount of total carotenoids, followed by tomato. Carrot has the lowest amount of TC but not significantly different with that of corn (table 1).

The researcher also tried to compare the obtained total carotenoid content from that of literature but no specific literature can be found listing the ranges of total carotenoid content specific for each plant sample. Rodriguez-Amaya¹¹ listed some foods with the amount of their total carotenoids but proved to be incomplete¹¹. The researcher found literatures that determine the total carotenoid content of the individual plant samples that is worthy to note in this paper.

Narvaez *et al.*¹³ presented some preliminary results of carotenoid synthesis in *B. orellana*, the plant where annatto came from. In this study, total carotenoids were quantified and found to be 142 to 10 526 mg/kg (µg/g) for different parts of the plant. Also, Matejkova and Petrikova examined differences between cultivars and the effect of storage regarding the carotenoid and vitamin C content in carrots. They used six carrot cultivars from early to late ones. Observed carotenoid content for carrots ranged from 60 to 134 mg/kg (µg/g)¹⁴.

Egesel *et al.* observed a variation of carotenoid levels among different genotypes of corn due to its concentration in parents. They also performed total carotenoid analysis and found that for nine common corn genotypes, carotenoid ranges from 13 – 128 µg/g dry weight (DW)¹⁵. In the study of Gama *et al.*, the carotenoid content of fresh tomato, tomato pulp and tomato

ketchup by high performance liquid chromatography was examined. They reported that tomatoes contain 67-71 µg/g of carotenoids on a fresh weight basis⁷.

It can be noted that the obtained measurement of TC content for annatto and corn is in the range reported in the literature. Data for carrot and tomato are just a little bit closer to the literature. These data confirmed what Dias *et al.*¹² reported in their paper that carotenoids vary qualitatively and quantitatively from food to food, within each species, food variety, and maturity.

Rodriguez-Amaya¹¹ in her book "A Guide to Carotenoid Analysis in Foods" listed the factors affecting carotenoid content such as cultivar or variety. Included in this list as factors affecting carotenoid content are the part of the plant consumed in food, the stage of maturity, climate or geographic site of production, harvesting and postharvest handling, processing and storage.

Thin Layer Chromatographic Analysis: The solvent system used in the thin layer chromatography (TLC) was 5% methanol in xylene according to the method of Rodriguez-Amaya (2001) with some modifications. The spots were visualized using iodine vapor and ultraviolet (UV) lamp. The spots on the TLC plates were marked using pencil. TLC results showed a diversity of phytochemicals contained in the four plant samples.

There were 11 spots identified in annatto, 4 spots in carrot, 3 spots in corn, and 7 spots in the tomato extracts (table 2). Retention factor (*R_f*) values obtained ranged from 0 to 0.98 which show spots were of differing polarities for each plant extract. The lowest *R_f* value of 0.00 means that the spot is a very polar compound. Polar compounds have great affinity for the polar silica gel plate. These spots could be oxygenated carotenoids. Some examples of oxygenated carotenoids are zeaxanthin, lutein, spirilloxanthin, echinenone, and antheraxanthin¹⁶. This means that plant sample extracts may contain these carotenoids. The spot with *R_f* value of 0.98 shows it has lowest polarity among the spots.

Table 2 also revealed the presence of similar spot in the samples which could imply the presence of similar compounds in the samples tested. For example, annatto and carrot has similar spot with *R_f* value of 0.08; annatto and corn with *R_f* value of 0.20, and annatto and tomato with *R_f* value of 0.42. *R_f* values of 0.00 and 0.98 were found present in all the samples. Those spots which have high *R_f* values indicate their strong affinity for the mobile phase which has greater proportion of nonpolar solvent, xylene. Thus the characteristic of this spot shows it to be nonpolar, more probably a carotenoid hydrocarbon like β-carotene.

The spots may be compounds of carotenoids. According to Rodriguez-Amaya (2001), monohydroxy carotenoids will be situated in the middle, trihydroxy carotenoids will remain in the origin, and dihydroxy carotenoids will be located between the

other two groups. High performance liquid chromatography and other methods can verify the identity of carotenoids.

Table – 2

Rf values of the plant samples separated by thin layer chromatography

Plant Samples	Annatto	Carrot	Corn	Tomato
Rf values	0.00	0.00	0.00	0.00
	0.08	0.08	0.20	
	0.20	0.23		
	0.25			
	0.32			
	0.42			
	0.53			
	0.63			
	0.70			
	0.87			
	0.98	0.98	0.98	
			0.42	
			0.50	
			0.58	
			0.67	

Antibacterial Assay: Shown in tables 3 and 4 are the results of antibacterial assay of the four plant sample extracts towards *Staphylococcus aureus* and *Escherichia coli*, a gram positive and gram negative bacterium respectively.

The highest antibacterial activity against *S. aureus* was observed in Streptomycin, the positive control, with mean zone of inhibition of 34.67 mm. No zone of inhibition was recorded in the negative control. All the plant extracts except corn extract were able to inhibit the growth of *S. aureus* which ranged from 7.50 to 9.17 mm (figure 1).

Table-3

Mean zone of inhibition (mm) of annatto, corn, carrot, tomato extracts against *Staphylococcus aureus*

Plant samples	Zone of inhibition (mm)
Annatto	9.17 ^b
Carrot	7.83 ^c
Corn	0.00 ^d
Tomato	7.50 ^c
Streptomycin (positive control)	34.67 ^a
Ethanol (negative control)	0.00 ^d

Note: Means having the same letter of superscript in the same column are not significantly different from each other at 5% level of significance.

Analysis of variance revealed significant differences among the extracts and controls in their ability to inhibit the growth of *S. aureus* at 5% level of significance. Duncan's Multiple Range Test (DMRT) shows that the mean zone of inhibition of annatto extract is significantly different from Streptomycin (positive control). However, when the mean zone of inhibition of the extracts were compared statistically, they were found to be significantly different from each other. Annatto has the highest

mean zone of inhibition but carrot and tomato extracts were found to have comparable effects against *S. aureus*. The result implies that all the extracts except corn have antibacterial property against *S. aureus*.

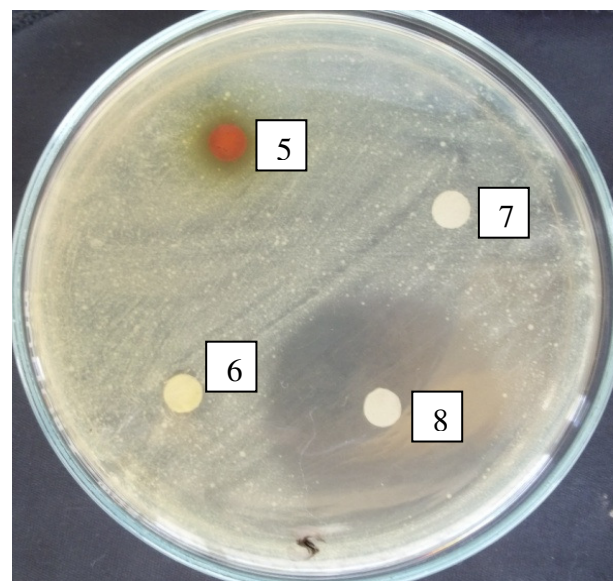
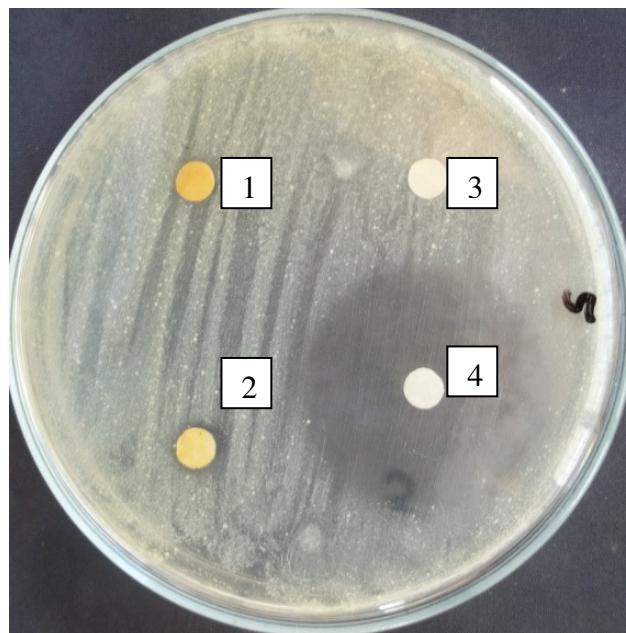


Figure - 1

Zone of inhibition of different treatments against *S. aureus* in nutrient agar. (1) tomato extract; (2) carrot extract; (3 and 7) ethanol; (4 and 8) Streptomycin; (5) annatto extract; and (6) corn extract

Table 4 presents the results of the antibacterial activity of the plant extracts against *E. coli*. No zones of inhibition were recorded in all the plant extracts. This means that extracts of annatto, carrot, corn, and tomato cannot inhibit the growth of *E. coli* as shown in figure 2.

Table - 4
Antibacterial activity of annatto, corn, carrot, and tomato extract against *Escherichia coli*.

Plant samples	Zone of inhibition
Annatto	-
Carrot	-
Corn	-
Tomato	-
Streptomycin (positive control)	+
Ethanol (negative control)	-

Note: (-) – no zone of inhibition was observed, (+) – zone of inhibition was observed

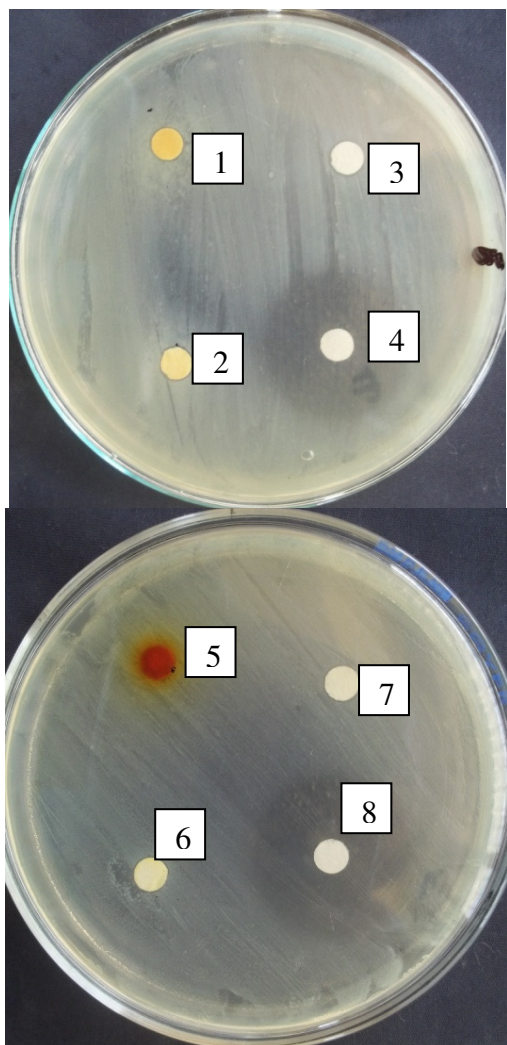


Figure-2

Antibacterial activity of different treatments against *E. coli* in nutrient agar. (1) tomato extract; (2) carrot extract; (3 and 7) ethanol; (4 and 8) Streptomycin; (5) annatto extract; and (6) corn extract

According to Nester, *et al.*, the components of the cell wall serve as the basis in differentiating gram positive and gram

negative bacteria¹⁷. Gram positive bacteria have cell wall as their outer covering (except for gram positive bacteria with capsules) while gram negative bacteria have an additional lipopolysaccharide that covers their cell wall. This means that gram negative bacteria have stronger defenses or protective layer than gram positive bacteria. Furthermore, family of phenolic compounds and tetraprenoids like tannins and carotenoids when present in large amount can break the cell walls of bacteria. On the other hand, the presence of extra defenses in gram negative bacteria specifically lipopolysaccharide may inhibit the effect of this phenolic compounds that is why annatto, carrot, corn and tomato extracts have no antibacterial activity for *E. coli*.

A correlation analysis was also made between total carotenoid content of the plant samples and their antibacterial activity. Annatto extracts with the highest total carotenoid content exhibited also the highest observed zone of inhibition for *S. aureus*. However, there was no trend observed for carrots, corn and tomato.

Conclusion

Annatto, carrot, corn, and tomato extracts has 931.30, 102.90, 123.90, and 192.23 µg/g of total carotenoids, respectively. Also, TLC results showed two spots were similar for all plant samples (*R_f* - 0.00, 0.98). These spots were found to be very polar and nonpolar. Moreover, annatto, carrot and tomato extracts exhibited antibacterial property for *S. aureus*, a gram positive bacterium. The plant extracts cannot inhibit the growth of *E. coli*, a gram negative bacterium. Annatto extract which has the highest total carotenoid content also exhibited the mean zone of inhibition for *S. aureus*.

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