

# ***In Vitro* Evaluation Of Biological Activity Of Anthocyanin Based Lipstick Formulations**

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## **ABSTRACT**

The growing market demands for health-promoting ingredients, as well as a growing concern over the use of synthetic colorants in consumer products, highlights the need for alternative coloring ingredients in cosmetic products. Anthocyanins, a class of flavonoids, have been reported as potent antioxidants, acting as anti-inflammatory, anti-carcinogenic compounds and as natural colorants in the food industry. However, their application in cosmetic products as active ingredients has scarcely been previously reported. Some of their reported benefits may be seen when applied topically in an appropriate skin-penetrating vesicle, such as liposomes. The objective of this study was to evaluate the use of anthocyanins as bioactive colorants in lipstick formulations. The hypothesis was that by targeting anthocyanin sources known for their high stability and reported health benefits, that these properties would also be observed in cosmetics. Formulations were tested for their ability to absorb UV light, free radical scavenging ability against DPPH, and inhibition of melanin production by tyrosinase. All formulas showed increased UV absorption over the lipstick base, with sources acylated with cinnamic acid exhibiting the highest in vitro SPF(UV-B) values. All formulas exhibited high inhibition of DPPH free radicals, and melanin production by tyrosinase, above that of the controls, BHT and kojic acid, respectively. Moreover, their activity was determined to be at physiologically relevant levels for lipstick applications. Our results suggest the potential for anthocyanins to be

used as biological active ingredients in cosmetic formulations by acting as antioxidants, UV-protection, and anti-aging compounds.

**Key Words:** anthocyanins, UV absorption, antioxidants, tyrosinase, cosmetics

## INTRODUCTION

Anthocyanins are water-soluble flavonoids responsible for many of the reds, blues and purple colors found in plants. In nature they occur primarily as one of six aglycones with various glycoside attachments: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Wu 2014). The variations in their chemical structure occur at the 3' and 5' positions of the B-ring and acylation of the sugar moiety with various aromatic and aliphatic acids may also occur. The biological activity of anthocyanins is largely dependent on these variations in their chemical structures.

The intense and attractive colors produced by anthocyanins have prompted interest in their uses as colorants for the food industry (Wrolstad and Culver 2012). Their potential to act as powerful antioxidants and use in disease prevention has also been gaining increased attention (Jing and Giusti 2014). They have long been considered as strong antioxidants, with the ability to scavenge free radicals and terminate chain reactions demonstrated in many in vitro assays (He and Giusti, 2010; Massa 2007). Their protective effects against oxidative stress-induced damage and regulation of redox-signaling pathways have also been demonstrated (Ramirez-Tortosa *et al* 2001; Shih *et al* 2012; Hwang *et al* 2011).

In addition, recent investigations into anthocyanins potential to prevent oxidative damage to the skin by UV-induced erythema, skin cancer, and photoaging have also demonstrated a

protective effect in vitro and in vivo (Chan *et al* 2010; Bae *et al* 2009; Tsoyi *et al* 2008; Hsu *et al* 2012).

Unabsorbed anthocyanins have also been shown to potentially act as chemopreventive topically in the gastrointestinal tract by preventing oxidative damage to the mucosal lining (Gee and Johnson 2001). This topical activity may also translate to similar benefits when applied to the skin in an appropriate vesicle, such that they are able to react with damaging reactive oxygen species (ROS) (Montanari *et al* 2013). A recent study demonstrated their ability to trigger a regenerative effect on the skin (Bojanowski 2013). Moreover, anthocyanins have been shown to improve psoriatic lesions in vitro and alleviate atopic dermatitis in vivo (Crisan *et al* 2013; Kim and Choung 2012).

Few studies have investigated the protective effects of anthocyanins when incorporated into matrices for topical delivery. However, two recent studies positively demonstrated the biological activity of anthocyanins when concentrated onto protein-rich matrices (Plundrich *et al* 2013) and when incorporated into ultradeformable liposomes (Montanari *et al* 2013).

The aim of this study was to investigate the biological activity in vitro of anthocyanins when incorporated into lipstick formulations as a source of color. The anthocyanins used were selected based on our previous studies investigating the color comparisons to commercially available lipsticks and stability of various anthocyanins sources within lipstick emulsions.

## **MATERIALS AND METHODS**

### *Materials*

Elderberry, purple carrot, purple sweet potato, and red radish dried extract were provided by DD Williamson & Co., Inc. (Louisville, KY), and the purple corn and red grape skin dried

extracts were provided by Artemis International (Fort Wayne, IN). The base of the lipstick formulations was purchased from MakingCosmetics, Inc. (Snoqualmie, WA). Black lip balm containers were purchased from a local company, Bulk Apothecary (Streetsboro, OH). Compounds used were gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), mushroom tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), and kojic acid, purchased from Sigma Aldrich (St. Louis, MO). Reagents used were ethanol and methanol, and were purchased from Fisher Scientific Inc. (Fair Lawn, NJ).

## *Methods*

### *Lipstick Formulations*

Formulations were based on recommendations in the Society of Cosmetic Chemists (SCC) Monograph Number 8: Lipstick Technology (Barone *et al* 2002). All dried extracts were incorporated as 8% of the final weight (w/w) of each lipstick formulation based on preliminary data. Dried extracts were initially weighed out and subjected to a grinding process with a mortar and pestle prior to being added to the lipstick manufacturing. Formulations underwent a wet grinding process in which castor oil was used at a 1:3 ratio (pigment:oil) and silica was included at 1% of the final weight (w/w), to increase uniformity in the final products. Initially, the lipstick base was weighed and placed in a water bath at 70°C with gentle stirring until completely melted. The pre-ground dried extracts were then poured directly into the hot lipstick base and gently stirred until uniform color was achieved. The lipstick formulas were then poured directly into the lip balm containers and allowed to cool at 4°C until completely solid.

### *Total Phenolic Content*

The Folin Ciocalteu method was used to estimate total phenolic content of the formulas containing anthocyanins, based on the methods described in the literature (Waterhouse 2000). 25mg of each formula was dissolved in 50mL of methanol, and briefly sonicated. Gallic acid was used as a positive control and gallic acid solutions were made in the following concentrations: 50mg/L, 100mg/L, 250mg/L, and 500mg/L. These concentrations were used to determine a standard curve for the test.

The Folin Ciocalteu reagent was kept at 4°C in the dark until the test was ready to be performed. 20µL of the formula-methanol solutions, the gallic acid solutions, or distilled water were pipetted into 3mL cuvettes, in triplicate. 1.58mL of distilled water was then pipetted into each cuvette. The Folin Ciocalteu reagent was then pipetted at a volume of 100µL into each cuvette and vigorously mixed by pipetting. 300 µL of sodium carbonate solution was then added to each cuvette after a rest period between 1-8minutes. The cuvettes were then equilibrated in the dark at room temperature for two hours. After the equilibration time, absorbance readings at 765nm were measured with using a spectrophotometer (Shimadzu UV-2450Spectrophotometer Kyoto, Japan).

Linear regression was used to determine a standard curve for the absorbance at 765nm of the gallic acid solutions ( $R^2= 0.99$ ). The standard curve was then used to determine the gallic acid equivalents (GAE) of the average of each sample absorbance. The results were reported as mg polyphenolic/liter of extract solution (GAE).

### *In Vitro UV Absorption and SPF Calculation*

*In vitro* sun protection factor for the lipstick formulas were determined based on the methods described by Sayre *et al* (1979) and Dutra *et al* (2004) with modifications based on the COLIPA revised method (Moyal *et al* 2012). Initially, 25mg of each formula was weighed and dissolved in 25mL ethanol and briefly sonicated in a water bath until no color was observed in the lipstick. 25mg of the lipstick base was also dissolved in 25mL ethanol by brief sonication in a heated water bath. 300µL of each solution was then pipetted into an ultraviolet microwell plate, in eight replications. Absorbance readings were carried out by a SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA) across the 290-400nm ultraviolet wavelength range, at 1nm increments and blanked against ethanol.

The absorbance values were then averaged and the standard deviation were calculated for each sample. The *in vitro* SPF values were determined according to the following formula:

$$SPF_{spectrophotometric} = CF \times \sum EE(\lambda) \times I(\lambda) Abs(\lambda) \quad (\text{Eq. 1})$$

Where CF= correction factor (10), EE(λ)= Erythema action spectrum (CIE 1987), I(λ) = Spectral irradiance received from the UV source, and Abs(λ) = spectrophotometric absorbance values at wavelength λ. The EE x I are constants and were determined by Sayre *et al* (1979). Results for the ultraviolet absorbance were graphed in 10nm increments between 290 and 400nm and standard deviations were included.

### *DPPH Free Radical Scavenging Assay*

The DPPH free radical scavenging assay was used to measure potential antioxidant capacity as described previously (Brand-Williams *et al* 1995) and (Prior *et al* 2005), with modifications described by Montanari *et al* (2013) for use with cosmetics. The methanolic

solutions containing the recovered pigments were used for the testing of antioxidant capacity. After the extraction from the formulas, 50mg of lipstick base was dissolved in 50mL methanol by brief sonication in a heated water bath, to be used as a positive control in the assay. BHT was also used as a positive control, and 50mg was weighed out and dissolved in 50mL methanol by stirring. Dried extracts were also weighed out at their respective amounts in each formula (8% w/w) based on their monomeric anthocyanin content. Weighed extracts were dissolved in methanol at a 1mg/mL (w/v) amount, and were used to account for the absorbance of anthocyanins at 515nm. DPPH was kept in the dark and at 4°C until needed in the assay. 2.5mg of DPPH was weighed and dissolved in 250mL methanol, still kept in the dark. Additionally, 5mg of DPPH was weighed and dissolved in 250mL methanol and subsequent serial dilutions were made to obtain a standard curve for DPPH based on concentration.

The DPPH methanol solution was then pipetted into 5mL plastic cuvettes at a volume ranging from 3.8-3.86mL. Then, 0.2-0.14mL of the methanol solutions containing either BHT, the lipstick base, dried extract, or the anthocyanin formulas were pipetted into the cuvettes and mixed thoroughly by pipetting. 300µL of each cuvette was then transferred into a microwell plate, in triplicate. The DPPH methanol solution was also pipetted at 300 µL in triplicate to serve as a positive control. The microwell plate was then placed in a Molecular Devices SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA), and allowed to equilibrate for 30 minutes in the dark at 20°C. Absorbance readings at 515nm were read after the 30-minute equilibration time and blanked against wells containing only methanol.

The average of the absorbance readings were then used to determine the DPPH inhibitory percentage and IC<sub>50</sub> of each sample. The inhibitory percentage of each sample was determined based on the following equation:

$$I\% = \left( \frac{Abs_{(DPPH)} - Abs_{(Sample)}}{Abs_{(DPPH)}} \right) \times 100 \quad (\text{Eq. 2})$$

The IC<sub>50</sub> values are defined as the concentration necessary to inhibit 50% of the free radical (Montanari *et al* 2013). The IC<sub>50</sub> values of each sample were determined using linear regression of the absorbances at different concentrations (40,60,100 µg/mL). The readings for the anthocyanin formulas were corrected for the absorbance of their respective dried extract in the methanol solution. Samples were tested again after 4 weeks of storage at 4°C and new IC<sub>50</sub> values were calculated and compared to the original values.

#### *Anti-Tyrosinase Assay*

Tyrosinase inhibition assay was performed as previously reported (Plundrich *et al* 2013). 120µL phosphate buffer (pH 6.5, 50mM), 40µL of formula extract, and 2µL of mushroom tyrosinase (5 U) were gently mixed and incubated for 10 minutes in a 96-well plate. Absorbance was measured at 475nm on a Molecular Devices SpectraMax190 Plate Reader (Molecular Devices, Sunnyvale, CA). Each sample was blanked against a well containing all components except mushroom tyrosinase. Results were compared with the negative control (phosphate buffer) and positive control (kojic acid). The percentage tyrosinase inhibition was calculated as follows:

$$I\% = \left( \frac{Abs_{(Control)} - Abs_{(Sample)}}{Abs_{(Control)}} \right) \times 100 \quad (\text{Eq. 3})$$

Results are presented as the mean (n=8) ± S.D. IC<sub>50</sub> values were predicted using linear regression and are expressed in µg/mL.



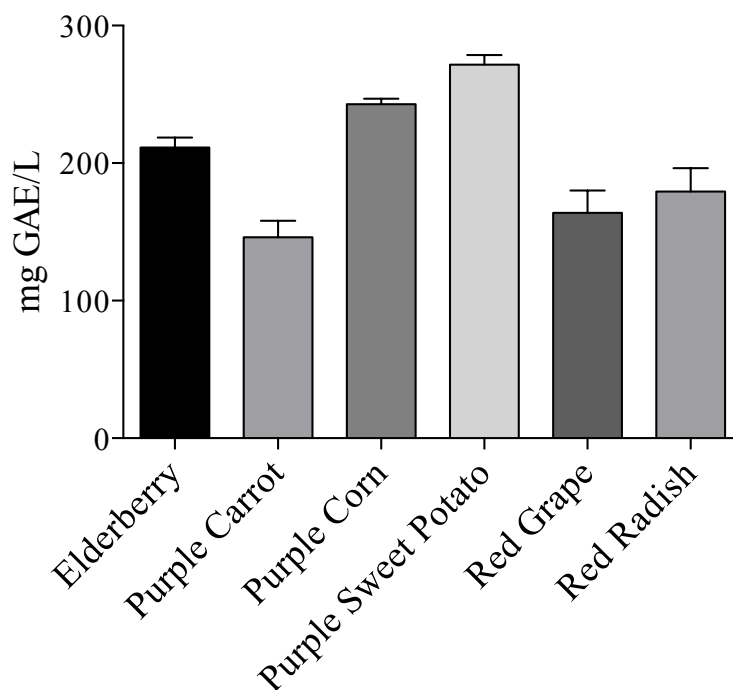
### Statistical Analysis

Results of Folin Ciocalteu, DPPH free radical scavenging assay, and anti-tyrosinase activity were analyzed using two-way ANOVA and regression modeling using Minitab Statistical Software ( $\alpha=0.05$ ) and GraphPad Prism Version 6.

## RESULTS AND DISCUSSION

### Total Phenolic Content

The amount of total phenolic content in the methanolic extracts was expressed as milligrams of equivalents of gallic acid in one liter (GAE) (Figure 1). GAE values were determined based on a calibration curve obtained from gallic acid ( $R^2= 0.99$ ).

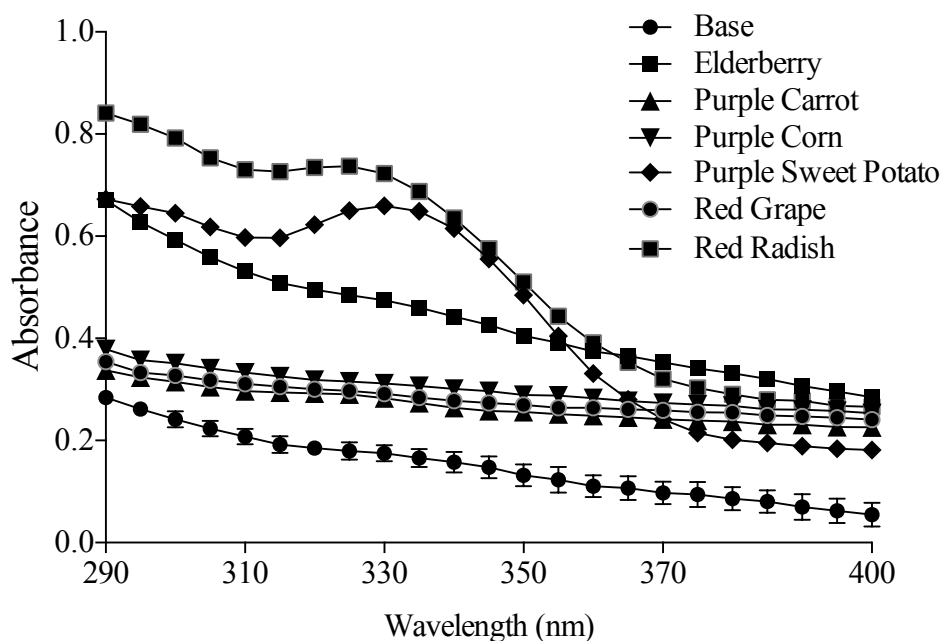


**Figure 1:** Total Phenolic Content of ACN-lipstick formulations in methanol, expressed as mg Gallic Acid Equivalents (GAE)/L ( $n=5 \pm$  SD).

Total phenolic content ranged from  $271.6 \pm 6.96$  mg GAE/liter extract for purple sweet potato to  $163.8 \pm 16.34$  GAE/liter extract for red grape skin.

#### UV Absorption and SPF Calculations

The ultraviolet light absorbance of each formula compared to that of the lipstick base alone, from 290-400nm, is shown in Figure 2.



**Figure 2:** UV absorbance of ACN-lipstick formulations compared to lipstick base in ethanol. Results are means ( $n=8 \pm SD$ ).

The formula that showed the highest ultraviolet absorbance was the red radish formula, across the 290-400nm range. The purple sweet potato formula also showed high absorbance across the ultraviolet range. Red radish, purple sweet potato and elderberry showed absorbance values at or above 0.5 for all wavelengths in the UVB (290-320nm) range (Table 1). All formulas showed greater absorbance than that of the lipstick base alone along the entire

ultraviolet spectrum, although absorption was higher in the UVB range than UVA range for all formulas tested. All formulas showed statistically significant differences when compared to the base.

**Table 1:** Calculated *in vitro* UVB sun protection factor (SPF) values of ethanolic extracts of ACN-lipstick formulations compared to the lipstick base. Calculations based on absorbance values in the ultraviolet wavelength range of 290-320nm.

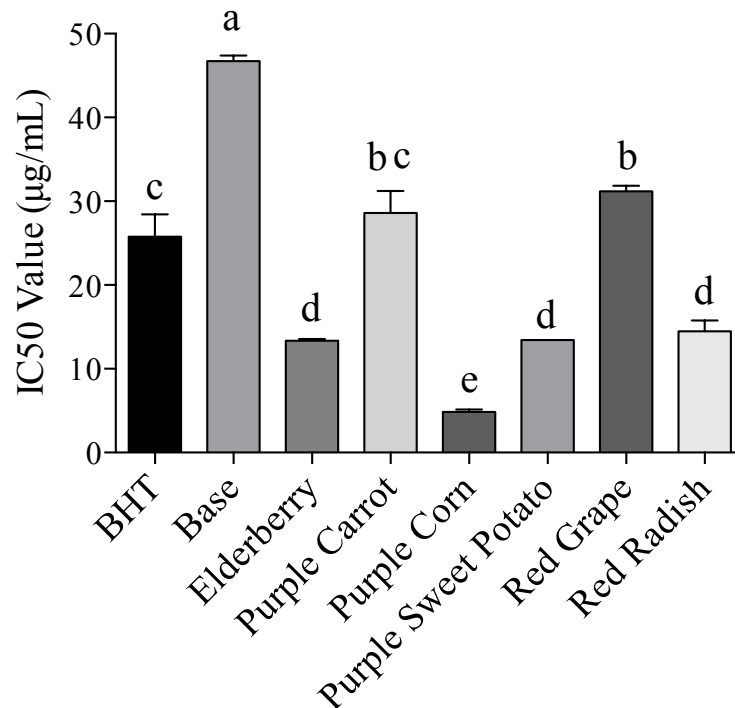
LIPSTICK FORMULATION	CALCULATED UVB SPF
Base	8.19
Elderberry	13.85
Purple Carrot	11.27
Purple Corn	11.62
Purple Sweet Potato	14.44
Red Grape	11.39
Red Radish	15.84

The calculated *in vitro* UVB sun protection factor (SPF) values of the ethanolic extracts of the formulas are shown in Table 1. Values followed the same pattern as that of the absorbance values in Figure 2. It was found that the calculated SPF values were highest for red radish, purple sweet potato, and elderberry and lowest for red grape skin, and purple carrot; however, all formulations showed an increase in absorbance over that of the base alone. The increased UV absorption by red radish and purple sweet potato may be due to the addition of acylation by cinnamic acids, which are known to absorb in the UV range (Crisvert and Salvador 2007). These results may suggest a future role for these extracts as photoprotective ingredients in topical formulas.

### *DPPH Free Radical Scavenging Ability*

The inhibitory percentages of each formula extract against DPPH at three concentrations (40,60,100 $\mu\text{g/mL}$ ) were determined to calculate the  $\text{IC}_{50}$  for each formulation. Inhibitory ability followed the same pattern at all three concentrations. The purple corn and elderberry formulas showed the highest inhibition of DPPH at all three concentrations while the grape skin and purple carrot formulas showed the lowest inhibition of DPPH at all three concentrations. All formula inhibitory values were significantly different than that of the lipstick base alone when analyzed by one-way ANOVA ( $p \text{ value} \leq 0.05$ ). Interestingly, when the inhibitory percentages of the formulas were analyzed against that of their respective extract in methanol no significant differences were found at all three concentrations. These results may be suggesting that the anthocyanins are behaving in a similar manner to their reducing behavior in solution. Free radical scavenging capacity of each formula extract, expressed as  $\text{IC}_{50}$  values is shown in Figure 3.

The antioxidant activity in order from highest activity found was: the purple corn formula ( $4.87 \pm 0.28 \mu\text{g/mL}$ ), elderberry formula ( $13.35 \pm 0.21 \mu\text{g/mL}$ ), purple sweet potato ( $13.44 \pm 0.01 \mu\text{g/mL}$ ), red radish ( $14.49 \pm 1.30 \mu\text{g/mL}$ ), purple carrot ( $28.59 \pm 2.65 \mu\text{g/mL}$ ), red grape skin ( $31.18 \pm 0.68 \mu\text{g/mL}$ ), and finally the base lipstick formula ( $46.72 \pm 0.67 \mu\text{g/mL}$ ). All formulations were significantly different when compared to the base formula. All formulas, except purple carrot and red grape were significantly different when compare to the positive control, BHT ( $25.78 \pm 2.66 \mu\text{g/mL}$ ).



**Figure 3:** IC<sub>50</sub> (µg/mL) of radical scavenging against DPPH for methanolic extracts of ACN-lipstick formulations compared to BHT and lipstick base (n=5 ± SD). Significant differences denoted by different letters above bars.

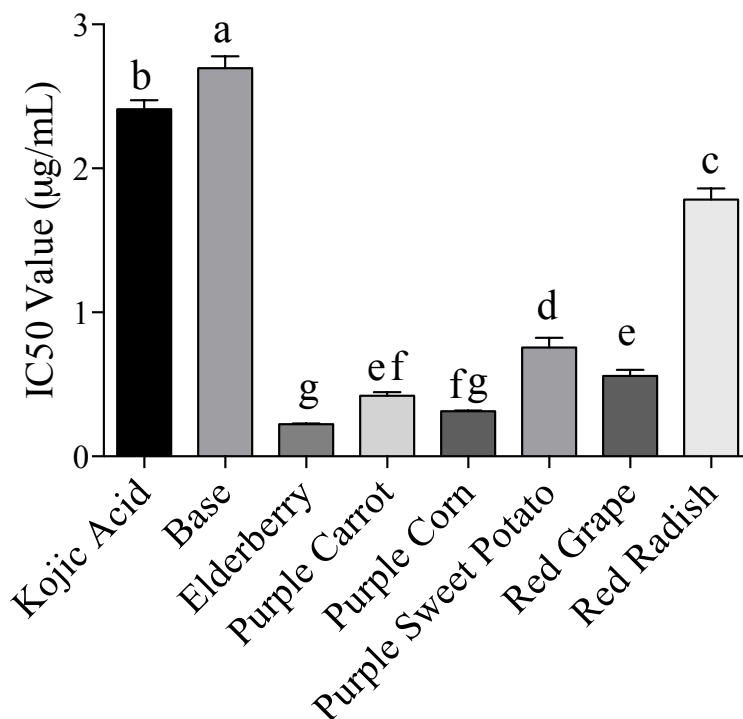
Overall, the antioxidant capacity values found were in agreement with the amount of total phenolic content discussed above. Purple corn, elderberry and purple sweet potato would be expected to have the highest antioxidant activity due to their higher total phenolic content. Inversely, the grape skin formula and purple carrot would be expected to have lower relative antioxidant activities, based on their phenolic content.

Samples were tested again after four weeks of storage at 4°C and inhibitory percentages and IC<sub>50</sub> values were compared to the fresh sample values. There was no statistical significance (p-value ≤ 0.05) between the values of the fresh samples versus the four week old samples.

### *Anti-Tyrosinase Activity*

Eluates from the base lipstick and the anthocyanin-lipstick formulations were tested for inhibition of L-DOPA oxidation against mushroom tyrosinase. IC<sub>50</sub> values (μg/mL), or the amount of the extracts necessary to inhibit 50% of the mushroom tyrosinase, were then predicted using linear regression and compared to the positive control, kojic acid (Figure 4). In general, all extracts showed the ability to inhibit tyrosinase at similar or lower concentrations than kojic acid (2.41 ± 0.06 μg/mL). The elderberry and purple corn, in particular, exhibited IC<sub>50</sub> values at 11-fold and 8-fold lower concentrations than that of the control. Results were analyzed using one-way ANOVA and all treatments were found to be statistically significant ( $p \leq 0.05$ ) when compared to controls.

IC<sub>50</sub> values (Figure 4) in order of highest inhibition were: elderberry (0.22 ± 0.06 μg/mL), purple corn (0.31 ± 0.01 μg/mL), purple carrot (0.42 ± 0.03 μg/mL), red grape skin (0.55 ± 0.04 μg/mL), purple sweet potato (0.75 ± 0.06 μg/mL), and red radish (1.78 ± 0.08 μg/mL).



**Figure 4:** IC<sub>50</sub> (µg/mL) against mushroom tyrosinase for ACN-lipstick formulations compared to kojic acid and the lipstick base. Results are presented as means (n=8) ±SD. Significant differences denoted by different letters above bars.

These results are in agreement with expected activity based on the structure of the anthocyanins present. Anthocyanins with a free hydroxyl groups at the 3' and 4' position of their B ring (cyanidin and delphinidin) would be expected to have the highest inhibition of tyrosinase (Parvez *et al* 2007). Conversely, it is believed that the substitution of a methoxy group on the 3' position of the B ring (peonidin, petunidin, and malvidin) causes steric hindrance of the hydroxyl group at the 4' position, decreasing inhibition potential. Acylation with cinnamic acids may increase inhibition (Parvez *et al* 2007). Acylation with malonic acid may also inhibit tyrosinase through complexation with the copper center of the enzyme. In addition, di-glucoside attachments at the 3 and 5 position of the A ring are believed to decrease inhibition through steric hindrance. Therefore the anthocyanin sources with cyanidin with one sugar

attachment at the 3 position (elderberry), as well as their acylated counterparts (purple carrot and purple corn), would be expected to have the highest inhibition of tyrosinase.

It should be noted that the results of IC<sub>50</sub> values in micrograms in a 1 milligram per milliliter solution are physiologically relevant to lipstick application. The average woman uses 24mg lipstick per day (Loretz *et al* 2005); therefore, the benefits against tyrosinase activity in cosmetic formulations can be achieved at relatively low concentrations.

## **CONCLUSIONS**

Anthocyanins incorporated into lipstick formulations were shown to retain their tested biological activity in vitro. All formulations showed the ability to act as photoprotective additives through UV absorption, especially those with cinnamic acid acylation. The anthocyanin formulations showed the capability to act as antioxidants, through scavenging of free radicals, in a lipstick matrix. In addition, the formulas showed proficient tyrosinase inhibition, which is a well-known source of melanin formation in the skin. The concentrations necessary to exhibit these activities were all well within physiologically relevant concentrations based on the average uses of lipsticks within the United States. These results show the potential for anthocyanins to be used as active ingredients within cosmetic formulations for these claim substantiations.



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