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IN VITRO ANTIOXIDANT ACTIVITY OF ANTHOCYANINS FROM THE PERICARP OF *TERMINALIA CATAPPA* L. FRUITS

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ABSTRACT

To elucidate the pigment molecules and antioxidant activity of anthocyanins from the pericarp of *Terminalia catapa* L. fruits. Method: Anthocyanins were isolated from the pericarp using standard method of isolation and their antioxidant activity was measured by *In vitro* methods *viz*., DPPH assay, metal chelating activity, reducing power and H_2O_2 scavenging assay. Results: The anthocyanins exhibited 70.11%, 64.66% and 70.6% inhibition at 100 µg/ml against DPPH, ferrous ion and hydrogen peroxide respectively. The reducing power was found to be concentration dependent. Conclusion: The anthocyanins isolated from pericarp of *Terminalia catapa* could be a source of natural pigment with antioxidant property.

KEY WORDS: anthocyanins, antioxidant activity, *Teminalia catappa*

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INTRODUCTION

Anthocyanins are an important group of naturally occurring pigments of red fruits such as red grape, elder, black currant, black berry, raspberry, black chokeberry, red cabbage, black carrot, purple corn, red raddish, cherries, plums, strawberries, red currants and purple sweet potato[1]. Anthocyanins are water soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium salts. Individual anthocyanins differ in the number of hydroxyl groups present in the molecules; the degree of methylation of these hydroxyl groups. They may appear red, purple or blue according to pH[2]. Health benefits associated with diets rich in anthocyanins are ascribed to multi-level biological activities including anti-oxidative, anti-inflammatory, anti-viral, vasoprotective, anti-angiogenic, anti-carcinogenic and also it is beneficial to health with potential physiological effects such as anti-neoplastic, radiation-protective, vasotonic, chemo and hepato-protective[3]. Supplements containing anthocyanin extracts or new synergistic blends were produced to improve human health. Hence, Anthocyanins have been a promising dietary compounds with an important role in human health[2]. Research on new viable sources of anthocyanins as natural food colorings is required to find alternatives with desirable stability, low cost and high tinctorial strength. There is a rising demand for natural sources of food colorants with nutraceutical benefits and alternative sources of natural anthocyanins are becoming increasingly important. Commercial anthocyanin colorants are mostly derived from fruits and vegetables[4]. The present study was aimed to expose the antioxidant activity of anthocyanins from *T. cattapa* fruits.

MATERIALS AND METHODS

Collection and preparation of plant material

The fruit of *Terminalia catappa L.* were collected from Coimbatore, Tamil Nadu, India and authenticated by Botanical Survey of India, Coimbatore, Tamil Nadu, India.

Extraction of Anthocyanin[5]

To obtain the anthocyanin extract, samples of 0.1 g of pericarp were extracted with 1 ml of acidified ethanol (up to pH 1 with hydrochloric acid) in a mortar, boiled at 95 $^{\circ}$C for 60 min and incubated at 4 $^{\circ}$C in the dark for 24 hours and centrifuged at 3000 rpm for 10 min.

Antioxidant activities

DPPH assay[6]

The hydrogen atom or electron donating abilities of the compounds were measured from the bleaching of the purple coloured methanol solution of 2, 2-diphenyl-1-picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable free radical, DPPH as a reagent. One thousand microlitres of diverse concentrations (20-500 µg/ml) of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The DPPH radical scavenging effect was calculated as inhibition of percentage (I %) using to the following formula

$I\ \% = (A_{Blank}-A_{Sample}/A_{Blank}) \times 100$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for various concentrations of the extract. Tests were conceded out in triplicate.

Reducing power assay[7]

0.5 ml of sample with different concentrations (20-500 µg/ml) was mixed with 0.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of a 1% potassium ferricyanide solution. The mixture was incubated in a water bath at 50ºC for 20 min. Subsequently, 0.5 ml of a 10 % (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. Finally, 0.5 ml of the supernatant layer solution was mixed with 0.5 ml of distilled water and 0.1 ml of 0.1% ferric chloride, and the absorbance of the reaction mixture was measured at 700 nm. Three replicates were made for each test sample. Increased

absorbance of the reaction mixture indicated increased reducing power of the sample.

Hydrogen peroxide radical scavenging (H_2O_2) assay[8]

A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20 - 100 µg/ml) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows

% Scavenged (H_2O_2) = ($A_0 – A_1$ / A_0) X 100

where A_0 is the absorbance of control and A_1 is the absorbance of the test. Ascorbic acid can be used as a positive control.

Metal chelating activity[9]

0.5 ml of the plant extract was mixed with 0.05 ml of 2mM $FeCl_2$ and 0.1 mM ferrozine. Total volume was diluted 2 ml of methanol. Then, the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was calculated using the formula giving below

$$\text{Scavenging effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A control is the absorbance of the ferrozine - Fe^{2+} complex and A sample is the absorbance of test compound. The percent of inhibition of absorbance at 562 nm was calculated.

Statistical analysis

The results of these investigations are means and SD of three measurements. Differences between groups were tested by two-way ANOVA. Linear regressions were also calculated. The P values of <0.01 were considered significant.

RESULTS AND DISCUSSION

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals, such as superoxide, hydroxyl peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$) radicals. Due to mitochondrial respiration, radicals derived from oxygen represent the most important class of radical species generated in living systems. Free radical metabolism seems to occupy a central and remarkably common position in causing potential biological damage, as the unpaired property, usually gives a considerable degree of reactivity to the free radical[10].

DPPH radical scavenging activity

DPPH radical is commonly used as substrate to evaluate antioxidant activity; it is stable free radical that can accept an electron or hydrogen radical to become a stable molecule. The reduction of DPPH radical was determined by the decrease in its absorbance at 517nm induced by antioxidants. Anthocyanins from *Terminallia cattapa* exhibited a significant ability to quench DPPH radicals (Table 1). The scavenging effect was increased with increasing concentration used in the test.

Reducing power

Reducing power is often used as an indicator of electron- donating activity, which is an important mechanism for testing antioxidative action[11]. Table 1 represents the reducing power of anthocyanin. It showed that anthocyanin had a dose dependent reducing power. The extract showed significant reducing power reflecting the reductive ability, which was measured by $Fe^{3+} \leftrightarrow Fe^{2+}$ transformation in the presence of test samples[12].

Metal chelating activity

Ferrozine could quantitatively form complexes with F^{2+}. In the presence of other chelating agents the complex formation was disrupted as the red color of the complex is decreased. Measurement of the rate of color reduction therefore allowed estimation of the chelating

effect of the coexisting cheater. Table 1 showed the chelating effects of anthocyanins on ferrous ions. The anthocyanin extract showed a sharp increase in scavenging ability in a concentration-dependent manner. The difference among the plant concentrations and control values were statistically significant ($p < 0.01$)[13]. Polyphenols can chelate pro-oxidant metal ions, such as iron and copper, thus preventing free radical formation from these pro-oxidants [14].

Hydrogen peroxide activity

Hydrogen peroxide enters the human body through inhalation of vapor or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage. It was estimated that the anthocyanins from *Terminalia catappa* shows higher percentage of inhibition against hydrogen peroxide with the lowest IC_{50} value of 29 µg/g. However, the antioxidant activity is greatly dependent on the chemical structure of anthocyanins and not all of them possess similar activities for scavenging diverse ROS and RNS. The antioxidant ability of anthocyanins depends on the basic structural orientation of the compound because the ring orientation will determine the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical as well as the capacity of the anthocyanin to support an unpaired electron[15]. Anthocyanins with the ortho-dihydroxyl groups have the potential to scavenge hydroxyl radicals through the inhibition of $HO^{\cdot}$ generation by chelating iron[16]. In addition to the degree and position of hydroxyl groups in the B ring on the antioxidant activity of anthocyanins, the degree and position of methoxyl groups also influenced the stability and reactivity of these pigments, consequently their antioxidant activities[17].

Table 1
Free radical scavenging activity of anthocyanins from T. catappa

Concentration µg/ml	Percentage of inhibition (I%)							
	DPPH scavenging activity		Metal chelating activity		Reducing power		H_2O_2 scavenging	
	T. catappa	Control (ascorbic acid)	*T. catappa*	Control (ascorbic acid)	*T. catappa*	Control (ascorbic acid)	*T. catappa*	Control (ascorbic acid)
20	41.10	21.93^{NS}	27.33	35.47^{NS}	1.570	0.973	30.6	45.00^{NS}
40	52.32	47.61**	28.26	39.78	1.698	1. 267	52.3	52.00
60	62.02	66.01**	40.00	46.31**	1.770	1.520	60.3	65.00**
80	68.50	71.26**	45.83	51.76**	1.837	1.610	63.0	69.00**
100	70.11	92.61**	64.66	65.20^{NS}	1.930	1.883	70.6	72.00**
SED	0.26		2.63		1.23		2.04	
IC_{50} µg/ml	37		86				29	

Significant NS – Not Significant

CONCLUSION

The anthocyanins from *T.catappa* showed excellent reducing capacity and free radical scavenging activity against both DPPH and H_2O_2. Therefore, anthocyanins as a type of natural pigment, safe non-toxic, rich in resources and with a certain biological activity, have great application potential in food, cosmetic as well as pharmaceutical fields.

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