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DIETARY ANTIOXIDANT AND IN VITRO ANTIOXIDANT EFFICACY FOR SOME INDIGENOUS DEEP WATER PADDY LAND RACES OF ASSAM (INDIA)

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ABSTRACT

Six deep water red rice land races were screened for total anthocyanins by pH differential spectrophotometric method. Highest anthocyanin content was found in *Surabhi* with 2.47 mg / g dry weight, followed by *Kakua* 2.05 mg/g. *Kakua* and *Kahijool* contain almost similar amount of anthocyanin. *Dal*, *Chakhao* and *Buruli* contain 1.77, 1.37 and 1.39 mg/g total anthocyanin respectively. Deffernces among the cultivars have been found to be significant(p-<0.01). *In vitro* antioxidant activities were determined by DPPH radical scavenging assay (RSA). There was a significant linear correlation between anthocyanin content and RSA (p<.05, r=o.929). *Surabhi* was found to be best with anthocyanin content 2.47 mg/g , RSA 90.685% and IC 50 value 1.66 mg/ml.

Key words.: Red rice (Deep water paddy), Anthocyanin, DPPH, RSA, IC 50

INTRODUCTION

Dietary antioxidants are gaining increasing importance in view of their health protective and health promoting effects (Wang 2003, Wu et al. 2004, Wojdylo et al. 2007). Although there are synthetic antioxidants, they can be utilized only as additive to processed food which is not affordable to the poorer section of the society. Moreover, growing evidences about biosafety aspects, particularly the adverse effects of synthetic antioxidants on human health (Namiki 1990, Pourmorad et al. 2006) has raised questions about their permissibility as food additive. Unlike this naturally occurring antioxidants are safer and acceptable as they occur as natural component of food. Although there are hundreds of diverse plant species with various types of antioxidants in varied amount; dietary antioxidants in paddy assume special significance because it is the staple food for nearly 60% of world population. Vast majority of them live in poor third world countries who can afford little for health protection and health promotion purpose. Unlike paddy, other antioxidant containing food plants like vegetables, leafy vegetables, fruits etc. are seasonal and not readily and regularly available. There are reports that rice with red kernel; generally referred to as 'red rice' contain substantial amount of anthocyanin (Loying et al. 2008) and it is this anthocyanin which is primarily responsible for red colouration. For a long time anthocyanins were considered as of little biological significance and of no practical utility although they are fairly widespread among diverse group of plants in different organs like leaf, flower, fruits etc. (Harborne 1976). Academic interest on anthocyanin revived with the reports that they are potent antioxidants (Tiwary 2001, Wu et al. 2004). Red rice is seldom marketed, but it is popular among the rural population of Kerala (Vanaja et al. 2008) and some other places like flood plains of Upper Assam etc. Initial reports for anthocyanin in rice (Loying et al. 2008) was reported for traditional varieties of deep water rice ('Bao dhan) and one cultivar of scented rice ('Joha dhan). But red colouration of paddy kernel is known to occur among diverse groups of traditional varieties. Deep water paddy land races are economic lifeline for people living in areas perennially inundated by flood in Upper Assam and Central Assam. The areas are mostly inaccessible and most of the most of the land races are scientifically unexplored. The present study was undertaken to analyze dietary antioxidant, antioxidant efficacy *in vitro* for some lesser known traditional land races of deep water paddy.

MATERIALS AND METHODS

Six indigenous land races of *Bao dhan* (deep water paddy) collected from few flood prone villages of Dhemaji sub-division of Upper Assam were taken for the present study. These are- *Dal, Chakhao, Buruli, Surabhi, Kakua, and Kahi jool.* All the varieties had red kernel with *Surabhi* being the darkest red of all.

Anthocyanin extraction.

The paddy grains were manually dehusked and 500 mg dry finely grounded samples were extracted with 80% methanol with 0.3% HCl. The samples were homogenized and centrifuged to obtain the supertanant. The residues were washed twice with the extraction medium and the supernatants were pooled. The final volume was made to 5ml with same extraction medium.

Analytical procedures

Anthocyanin estimation was carried out as per the protocol of Cheng and Breen (1991). 1 ml of extracts were diluted in 5 ml of two different buffers; 0.025 M potassium chloride pH = 1.0 and 0.4 M sodium acetate pH = 4.5, respectively. After 30 minutes of incubation at room temperature, absorption (A) was measured at λ 510 nm and 700 nm (Thermo Scientific, Aquamate Spetrophotometer, USA). All extracts were analyzed in triplicate. The difference in absorbance between pH values and wavelengths was calculated as follow:

$$Asp = (A_{510} - A_{700})\ pH1.0 - (A_{510} - A_{700})\ pH\ 4.5$$

The anthocyanin concentration of crude extract was calculated according to the following formula and expressed as cyanidin-3-glucoside equivalents :

$$\frac{A \times MW \times DF \times 1000}{\lambda \times \varepsilon \times m}$$

A = absorbance of samples
MW = molecular weight of cyanidin-3-glucoside (449.2 g/mol)
DF = dilution factor of sample
ε = molar absorptivity (ε for cyanidin-3-glucoside as 15600 M-1 cm-1ε, Giusti et al. 1999]

λ = is the cuvette optical pathlength (1 cm)

m = is the weight of the sample (g)

IN - VITRO ANTIOXIDANT ASSAY BY DPPH RADICAL SCAVENGING ACTIVITY

Antioxidant efficacy of the individual sample was assayed by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and reducing power assay, DPPH reduction assay was carried out as per the method of Abe et al. (1998). For the DPPH test a methanolic extract of the sample was used. For this 300 mg dried powered sample was extracted with methanol. The mixture was homogenized by putting in magnetic stirrer for 72 hours. There after the mixture was centrifuged at 5000 rpm for 10 minutes and the supernatant was collected. The residue was washed twice with methanol and supernatants were pooled. Total volume of the supernatant was made upto 25 ml by adding required amount of methanol.

COLOUR REACTION AND SPECTROSCOPY

For the colour reaction 200µl extract was taken to which 1.8 ml methanol was added. This was followed by addition of 2 ml DPPH solution (0.1 mM DPPH prepared with methanol). The mixture was now incubated at dark for 30 minutes at room temperature. The negative control comprise of 200µl methanol which was processed in the similar manner as that of individual sample.

The reduction or non reduction of the purple colouration of the DPPH in negative control as well as individual sample were measured in a UV-Vis spectrophotometer (Aquamate Plus Thermo Scientific) at a wave length of 517nm. For scale setting methanol was used as blank. The absorbance value for negative control (unbleached DPPH) was considered as baseline value. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration, using the equation:

$$\%RSA = [(A_0-A_S)/A_0] \times 100$$

Where, A_0 is the negative control (unbleached DPPH) and A_S is the absorbance of individual sample.

For determination of IC 50 value , for each sample a series of gradient solutions were prepared in the range of 100µl to 1000µl with an increment of 100µl and absorbance values were recorded (Fig. 1). The values were plotted on graph and IC 50 values was computed taking negative control absorbance as baseline.

RESULTS AND DISCUSSION

Highest anthocyanin content was found in *Surabhi* with 2.47 mg / g of dry rice powder, followed by *Kakua* 2.05 mg/g. *Kakua* and *Kahijool* contain almost similar

amount of anthocyanin. *Dal*, *Chakhao* and *Buruli* contain 1.77, 1.37 and 1.39 mg/g total anthocyanin respectively (Table 1). One way Anova shows p-value (<0.01) indicating significant variation among the land races. In addition to one way analysis of variance which gives a general impression about significance a post hoc test was carried out for planned comparison among particular means (Table 2)

Table 1: Anthocyanin content among the six landraces of deep water paddy and their radical scavenging activity

CULTIVAR	**Anthocyanin mg/g dry wt. ± SEm**	**Abs λ 517**	**%RSA ± SEm**
DAL	1.77 ± 0.029	0.431	55.855 ± 0.08
CHAKHAO	1.37 ± 0.019	0.657	32.753 ± 0.11
BURULI	1.39 ± 0.022	0.539	44.831 ± 0.12
SURABHI	2.47 ± 0.030	0.091	90.685 ± 0.06
KAKUA	2.05 ± 0.021	0.261	73.285 ± 0.06
KAHI JOOL	2.01 ± 0.023	0.105	89.252 ± 0.18
WHITE RICE	Trace	0.780	20.163 ± 0.09

p-value (<0.01) , (p<.05, r=0.929)

Table 2: Post hoc test for planned comparison among particular means.
Dependent Variable : anthocyanin

(I) Cultivar	**(J) Cultivar**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
DAL	CHAKHAO	.44000*	.05044	.000	.2706	.6094
	BURULI	.40000*	.05044	.000	.2306	.5694
	SURABHI	-.65000*	.05044	.000	-.8194	-.4806
	KAKUA	-.19667*	.05044	.020	-.3661	-.0272
	KAHI JOOL	-.22000*	.05044	.009	-.3894	-.0506

(I) Cultivar	(J) Cultivar	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CHAKHAO	SURABHI	-1.09000*	.05044	.000	-1.2594	-.9206
	KAKUA	-.63667*	.05044	.000	-.8061	-.4672
	KAHI JOOL	-.66000*	.05044	.000	-.8294	-.4906
	DAL	-.40000*	.05044	.000	-.5694	-.2306
	BURULI	.04000	.05044	.963	-.1294	.2094
BURULI	SURABHI	-1.05000*	.05044	.000	-1.2194	-.8806
	KAKUA	-.59667*	.05044	.000	-.7661	-.4272
	KAHI JOOL	-.62000*	.05044	.000	-.7894	-.4506
	DAL	.65000*	.05044	.000	.4806	.8194
	CHAKHAO	1.09000*	.05044	.000	.9206	1.2594
SURABHI	BURULI	1.05000*	.05044	.000	.8806	1.2194
	KAKUA	.45333*	.05044	.000	.2839	.6228
	KAHI JOOL	.43000*	.05044	.000	.2606	.5994
	Dal	.19667*	.05044	.020	.0272	.3661
	CHAKHAO	.63667*	.05044	.000	.4672	.8061
KAKUA	BURULI	.59667*	.05044	.000	.4272	.7661
	SURABHI	-.45333*	.05044	.000	-.6228	-.2839
	KAHI JOOL	-.02333	.05044	.997	-.1928	.1461
	DAL	.22000*	.05044	.009	.0506	.3894
	CHAKHAO	.66000*	.05044	.000	.4906	.8294
KAHI JOOL	BURULI	.62000*	.05044	.000	.4506	.7894
	SURABHI	-.43000*	.05044	.000	-.5994	-.2606
	KAKUA	.02333	.05044	.997	-.1461	.1928

*. The mean difference is significant at the 0.05 level.

Significant	p-value	significant/ Not significant
Dal & Chakhao	.000	Significant
Dal & Buruli	.000	Significant
Dal & Surabhi	.000	Significant
Dal & Kakua	.020	Significant
Dal & Kahijool	.009	Significant
Chakhao & Buruli	.963	Not Significant
Chakhao & Surabhi	.000	Significant
Chakhao & Kakua	.000	Significant
Chakhao & Kahijol	.000	Significant
Buruli & Surabhi	.000	Significant
Buruli & Kakua	.000	Significant
Buruli & Kahijol	.000	Significant
Surabhi & Kakua	.000	Significant
Surabhi & Kahijol	.000	Significant
Kakua & Kahijol	.997	Not Significant

Post Hoc Tests and Multiple Comparisons test shows that the pairs *Chakhao* and *Buruli* and *Kakua* and *Kahijool*, are not significant, all the other pairs are significant. It shows that anthocyanin content in *Dal* significantly differs from the other land races. Similarly, presence of anthocyanin in *Surabhi* significantly differs from the other varieties. For rest of the land races, it is significant with four varieties.

Based on DPPH reduction test it was found that Radical Scavenging Activity (RSA) was also highest in case of Suravi, which was almost 90% as against lowest in case of white rice with 20.16% The results and analysis show that RSA is linearly correlated with the anthocyanin content. IC 50 value was recorded 1.66 mg/ml in *Surabhi* and highest 10.374mg/ml in *Chakhao*(Fig. 1, Fig. 2). Moreover IC 50 value is also inversely proportinal to the anthocyanin content(Table 3). Based on these observation it can be attributed that anthocyanin of red rice cultivar (Bao Dhan) has a strong antioxidant property as reported by other workers. Earlier workers presented anthocyanin content in terms of absorbance value which gives a relative

value and not exact quantification. Recent development of the pH differential spectroscopic method enable quantification. The majority of quantitative analysis methods need calibration with high purity of the analyzed compounds. Since anthocyanins exist in plants as mixtures of several compounds with similar chemical properties and the purification process is complicated, therefore the high purity anthocyanins are expensive (Wrolstad et al. 2005). The pure anthocyanins are also very unstable and susceptible to degradation (Giusti and Wrolstad 2003). The pH differential spectroscopic method gives handy useful tools to calculate quantitative estimation using the molar absorption coefficient (ε) and molecular weight data from previously published works. In the present work data from anthocyanin content are in terms of quantity (mg/g dw) instead of absorbance value.

Table 3: IC 50 values for six landraces of deep water paddy based on DPPH scavenging Assay

CULTIVAR	Abs. range for DPPH reduction at λ 517	IC 50 (mg/ml)
DAL	0.735 0.388	7.77
CHAKHAO	0.733-.502	10.374
BURULI	0.778-0.504	9.72
SURABHI	0.418-0.059	1.66
KAKUA	0.631-0.180	4.62
KAHI JOOL	0.548 -0.086	2.76
WHITE RICE	0.787-0.698	-

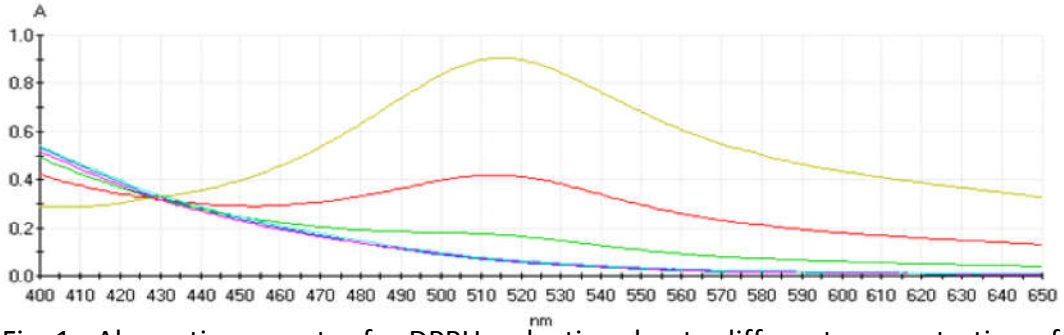

Fig. 1 : Absorption spectra for DPPH reduction due to different concentration of methanolic extract of cv. Surabhi.

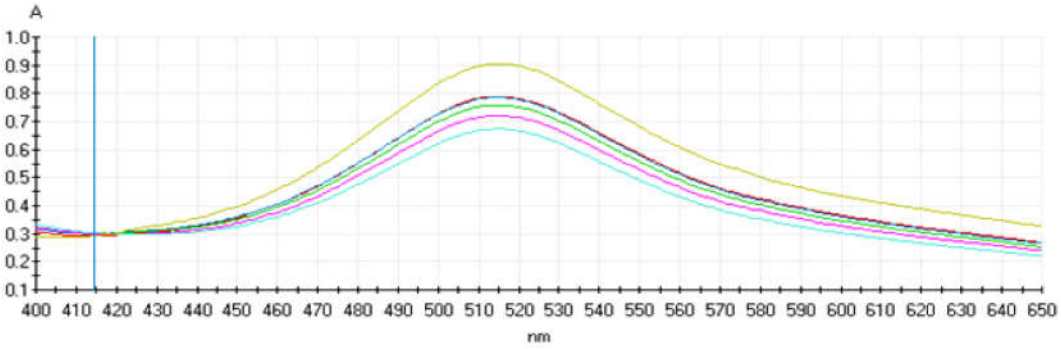

Fig. 1 : Absorption spectra for DPPH reduction due to different concentration of methanolic extract of commercial white rice

Studies by a number of workers have revealed that redness or blackness of coloured rice is primarily due to anthocyanin (Loying et al. 2008, Chakutou et al. 2012) Moreover it is the proportion of anthocyanin content, which is the determining factor for the degree of redness. Sompong et al. (2011) studied anthocyanin content of black rice varieties from Thailand, China and SriLanka and observed that black rice have comparatively higher anthocyanin than red rice where anthocyanin varied in the range of 0.33 – 1.38 mg/g. Similar findings were reported by Yodmanee et al. (2011) for red rice varieties of Thailand. In the present study anthocyanin content varied from 2.47 mg / g to 1.3mg/g. Therefore the findings of the present work are in agreement with earlier report. A comparison show that some red rice land races of Assam are superior to those of Thailand and SriLanka. In vitro antioxidant activities observed in the present study are comparable to those of earlier reports for red rice for Malaysia (Lum and Chong2012). Moko et.al (2014) working with coloured and non coloured rice varities of Indonesia found RSA% based on DPPH reduction in the range of 51.02 ± 1.10 - 88.29 ± 5.62% with IC 50 value in the range of $341.88 \pm 74.10\ \mu g/ml$ to $363.17 \pm 91.21\ \mu g/ml$. Rao et.al (2010) working with four rice varities of India reported that the cultivar Njavara had highest DPPH scavenging activities with an IC-50 value of 30.85 mg/ml. In the present study the DPPH scavenging activities varied in the range of 90% to 32% with IC 50 value in the range of 1.66mg/ml to 10.37mg/ml. The findings of the present study are in agreement with the earlier reports and some of the land races of Assam are superior with respect to antioxidant efficacy compared to those of other countries.

In vitro antioxidant activities may be for a number of dietary antioxidants present in the extract like phenolics, flavonoid, ascorbic acid, and many more apart

from anthocyanin and because of these sometime a definite co- relationship between a particular antioxidant and antioxidant activity is not observed (Subhasree et al. 2011). This is attributed to phytochemical diversity. In the present study a strong positive co-relation($p<0.05$; $r=0.929$) has been observed between anthocyanin content and RSA % indicating that in case of red rice antioxidant activity is primarily due to anthocyanin. Several workers have demonstrated that there is a positive co-relation between anthocyanin content of coloured rice and in vitro antioxidant activity (Chakuton et al. 2012, Henderson et al. 2012) which lend support to the present findings. Pengkumsri et al. (2015) employed several in- vitro assays viz lipid peroxidation, superoxide anion, nitric oxide assay for antioxidant activity and observed a positive co-relation between anthocyanin content and in vitro antioxidant activity.

Apart from dietary antioxidant and antioxidant efficacy, red coloured deep water paddy land races are known to be nutritionally very rich with high protein content (9% to 13%), lipid, carbohydrate and calorific value (Baruah et al. 2006, Loying et al. 2010) All these together with present study shows that the indigenous land races of deep water paddy (Bao Dhan) which are little explored , receive little attention; have in fact excellent nutritive and nutraceutical values.

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