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Antioxidant potential of processed *Vigna umbellata* (L.) seeds: An Indian underutilized legume

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Abstract

The raw and processed seeds of rice bean variety (RBL-6) grown in India were examined for bioactive compounds (total phenolic content, hydrophilic and hydrophobic phenols, o-dihydric phenols, flavonoids) and antioxidant potential (free radical scavenging capacity by DPPH, total antioxidant activity using ferric thiocyanate assay). One-way ANOVA revealed significant differences in phytochemical constituents in different methanolic extracts. Results concluded that phenolic contents in seed parts as well as in cooked cotyledons correlated ($p < 0.05$) significantly with antioxidant properties in most of the extracts. Hence, the methanolic extracts of seed coat showed highest phenolics (103.62 mg GAEg⁻¹) and highest scavenging efficiency against DPPH (0.17 mg/ml) as a consequence. The present study demonstrated that ricebean, an unexploited legume endowed with high bioactive constituents as well as antioxidant potential and thus serve as future nutraceutical source.

Keywords: *Vigna umbellata* (L.), Indian underutilized legume, processed seeds, ferric thiocyanate assay

Introduction

Several studies have shown a highly significant correlation between uptake of foods with high antioxidant potential such as fruits, vegetables, legumes and decreasing occurrence of severe diseases namely cancer, cardiovascular diseases, hypocholesterolemic effects, diabetes etc. (Nithiyanantham *et al.*, 2012) [1]. Based on the mechanism of antioxidant activity, poly phenolics are categorized as chain breaker, oxygen scavenger, metal in activator and peroxide decomposer. Legumes which play an important role as human food or animal feed are widely utilized for health perspective. Rice bean (*Vigna umbellata* L.) is one of the unexploited and underutilized legume crop widely grown in rice fields after the harvesting of paddy on residual soil moisture. Kaur and Kapoor (1991) [6] have studied the nutrient profiles in rice bean and reported that rice bean is comparable to commonly-consumed pulses such as black gram and green gram. The red rice beans are used to relieve oedema in some illnesses and are an integral part of traditional Chinese therapy. Rice bean as a grain legume is attracting attention throughout the world as a potential source of high-quality protein for the future for bridging the 'protein gap'. Hence, it is one example of the 'underutilized crops' or 'orphan crops' that have received attention over the last few years due to the still narrower species and genetic base in the global food supply. Although the nutritional value of rice bean seeds has been discussed in previous literature still information regarding phenolic content and antioxidant profile of seed material is scanty.

Most of the legumes need to be processed before consumption to improve their palatability. In spite of that, the processing may affect their antioxidant potential of food. Hence, the objective of this study was to evaluate the profile and content of phenolic compounds and their relation to the antioxidant capacity as affected by processing like de hulling, cooking etc. in rice bean grown in Haryana (India). Therefore, the present study has been aimed at:

1. To determine the chemical composition of whole seeds, seed coats, de-coated raw and cooked dal of rice bean.
2. To evaluate the anti-radical and antioxidant activity of the methanolic extracts of the above samples.

This study will provide much beneficial information for the food and nutraceutical industry from underutilized legume crop and serve as a good base for other researchers to investigate legume antioxidants in future research.

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Materials and Methods

Seed material

For experimental analysis, healthy seeds of rice bean var. RBL-6 were provided by the Pulses section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University (India). These seeds were cleaned to remove foreign materials, damaged seeds and immature seeds. After sorting, a major portion of seeds from each variety was dehusked to obtain seed coat and dal. Dehusked dal was divided into two sets. The first set was kept for extraction which constitutes intact dehusked seeds and the second part was cooked using distilled water in 1:10 (w/v) ratio at 100°C, dried at 55 °C. Thus, we obtained four samples of rice bean, 100 gm each for further analysis.

Preparation of extracts

For the preparation of extracts, the samples were ground using an electric grinder and converted into fine powder. Powdered samples were then extracted separately by refluxing for six hours using methanol. Extracts produced after refluxing were filtered and the filtrates were concentrated under reduced pressure on rotating evaporator at 40°C. These concentrated filtrates were stored at 5 °C for analyses of phytoconstituents.

Determination of total phenolic content

Determination of total phenolic content was done by Folin-Ciocalteu reagent using gallic acid as standard (Shahidi and Nacz, 2001). To a 50 ml volumetric flask 1.0 ml extract, 1.0 ml Folin-ciocalteu reagent (50% v/v) and 2.0 ml of Na₂CO₃ (20% w/v) were added and mixed and final volume was made to 50 ml. The mixture was allowed to settle for 30 minutes and then centrifuged at 6000 rpm for 5-7 minutes. After centrifugation, the solution was measured colorimetrically at 730 nm using Shimadzu UV-Vis spectrophotometer (UV-2600). A blank was also prepared by following same aforementioned procedure without a sample. After multiplication with the dilution factor, the concentration of phenolic content was expressed as equivalent to milligrams of gallic acid per gram of extract (mg GAEg⁻¹) by using the standard plot.

Determination of hydrophilic and hydrophobic phenolic contents

For the determination of hydrophilic and hydrophobic phenolic contents, 50 ml of crude extract was fractionated to obtain constituting hydrophilic and hydrophobic components. It was done by mixing the extract with deionized water and n-butanol (100ml each) in separating funnel as per Wettasinghe's method (2000). The mixture was then allowed to stand until separate layers visible. Separated layers were then concentrated using rotavapor at 40°C temperature. After measuring the weight of each fraction, the phenolic content of each fraction was determined as per Shahidi and Nacz method.

Preparation of standard curve of o-dihydric phenols

Preparation of standard curve was done by estimating o-dihydric phenols in methanol extracts by Arnow's method (Kim *et al.*, 2003) using catechol as standard. 0.4 ml of extracted solutions were added to 1 ml 0.5N HCl, 1 ml Arnow's reagent and 2 ml 1N NaOH. The final volume was made to 10 ml using double distilled water. The intensity of resulting orange red color was measured colorimetrically at 515 nm using a spectrophotometer. A standard curve was made by plotting absorbance against the amount of catechol

used (mg). A blank was also prepared by following same aforementioned procedure without extract.

Determination of flavonoids

The aluminum chloride colorimetric assay, as described by Zhishen *et al.* (1999) [20] with modification was used. Briefly, 1 ml of diluted (1:4) extracts were added. A blank solution using doubled distilled water was prepared. Then 0.3 ml 5% NaNO₂ was added to the testing samples, followed by 0.3 ml 10% AlCl₃, 2 ml of 1M NaOH and the total volume was made 10ml with dilution and mixed thoroughly. Then the absorbance was measured at 510 nm against blank. Total flavonoid contents were expressed as mg catechin equivalent per gram of the extract (mg CAE/g).

2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used to evaluate the antioxidant activity of the both the genotypes. The antioxidant response of extracts against DPPH free radical was estimated by the method of Hatano *et al.*, 1988. Methanol treated extracts were dried completely and weighed. The dried mass of methanol extracts was re dissolved in required volume of methanol to make the stock solution (1 mg/ml).

Different concentrations (0.1-1 mg/ml) were made by appropriate dilutions with 100% methanol from the stock solution. In extracts of different concentrations, 2.0 ml of DPPH solution (0.025 g L⁻¹ in 50% methanol) was added and the mixture was shaken and absorbance was measured at 515 nm at every 5 minutes interval until the reaction subsided. After 2 hours, the percent absorbance was declined corresponding to the percentage of DPPH scavenged which was an expression of antioxidant activity. During this process, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) with conc. 0.1-1.0 mg/ml were used as standard solutions having same preparatory method. Similarly, a control sample was also prepared. By using Microsoft Excel Software, a quadratic regression equation ($y = ax^2 + bx + c$) was obtained. By putting $y = 50\%$ in the equation $y = ax^2 + bx + c$; it was converted to the form $ax^2 + bx + c = 0$. IC₅₀ was calculated from the equation $ax^2 + bx + c = 0$ by using the formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (mg/ml)

Calculation

The calculation for DPPH scavenged (% DPPH^{*sc}) was done by following formulae:

$$\% \text{ DPPH}^*_{sc} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where A_{control} and A_{sample} represents the absorbance of control and sample. Based on the results obtained after calculation a graph was made by plotting percent DPPH free radical scavenging activity or inhibition percentage (y-axis) against extract concentration (x-axis).

Ferric thiocyanate (FTC) method

The FTC method was given by Kikuzaki and Nakatani (1993) [7]. This method was used for the evaluation of antioxidant activity of the extracts under study. After making required dilutions with methanol, 0.1 to 1 mg/ml of samples were made. After that, samples were mixed with 5 ml of linoleic acid emulsion and final volume was made to 10 ml using 0.2 M phosphate buffer (pH 7.0) and incubation was done at 37°C for 96 hours (4 days). After incubation aliquots of 0.1 ml were drawn from the incubated mixture after 24 hours interval and mixed with 30% ammonium thiocyanate, 20 mM ferrous chloride in 3.5% HCl and final volume was made to 10 ml with 75% ethanol and allowed to stand for 10 minutes. The color developed was measured colorimetrically at 500 nm using aspectrophotometer. For this experiment, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as standard solutions and by using quadratic regression equation ($y = ax^2+bx+c$) calculations were done as described earlier. A control mixture was prepared simultaneously following same procedure without the test sample.

Calculation

Observed antioxidant activity was expressed as:

Antioxidant activity (%) = $\{1 - (\text{increase in abs. of sample} / \text{increase in abs. of control})\} \times 100$

Table 1: Phytochemical contents and antioxidant activity of ricebean extracts.

Parameters	Seed Coat	Dehusked Dal	Cooked Dal	Whole Seed	BHT as Standard
Yield (%) of methanolic extracts	5.31	6.03	3.09	9.21	—
Total phenolics (mgGAE/g)	103.62	45.10	21.95	52.15	—
Total o-dihydric phenols mg COEg ⁻¹	42.35	30.57	9.92	32.32	—
Hydrophilic phenols (mgGAE/g)	53.09	32.23	9.82	31.26	—
Hydrophobic phenols (mgGAE/g)	50.53	12.87	12.12	20.88	—
Flavonoids (mgCAE/g)	25.32	33.66	15.80	30.19	—
DPPH radical scavenging assay EC ₅₀ (mg/g of extract)	0.17	0.35	0.43	0.25	0.41
Ferric thiocyanate (FTC) assay EC ₅₀ (mg/g of extract)	0.29	0.65	0.69	0.57	0.63

Values are mean of four replicates \pm standard deviation and means are different from each other by F-test ($p < 0.05$)

mg GAE/g- milligrams gallic acid equivalent/g of the extract

mg CAE/g- milligrams catechin equivalent/g of the extract

Estimation of ortho - dihydric phenolic content

Analysis of variance of means ($p < 0.05$) showed that there was the significant statistical difference between the o-dihydric phenolic content of all the extracts. It was observed that ortho-dihydric phenolic content was highest in seed coat extract (42.35 mg COEg⁻¹) analogous with the result obtained for total phenolics. Corresponding values for o-dihydric phenolic content in raw dal, cooked dal, whole seed extracts were 30.57, 9.92, 32.32 mg COEg⁻¹ which were statistically different from each other. For 100 g sample, the o-dihydric phenols were observed as 0.22 % in seed coat extract, 0.18 % in raw dal extract, 0.03 % in cooked dal extract and 0.29 % in whole seed extract. This data was in close agreement with total phenolics (305 mg/100g) determined by Gujral *et al.*, 2011 who reported 25% decrease in total phenolic on cooking.

Estimation of hydrophobic and hydrophilic phenolic content

In ricebean extracts, it was observed that total phenolics reported in seed coat extract was 103.62 mg GAEg⁻¹ and it consisted of 49% (w/w) and 51% (w/w) of hydrophobic and

Statistical analysis

Four replications of each sample were used for statistical analysis for minimizing random experimental error. Values obtained were expressed as mean \pm S.E. Both one way and two-way analysis of variance (ANOVA) and F-test were carried out to assess significant differences in between means ($p < 0.05$). Correlation analyses of polyphenolic composition and their antioxidant activities were carried out using Pearson correlation program in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

Estimation of Phyto-constituents and Antioxidant Activities

Estimation of total phenolic content

As per results analyzed (Table 1) for estimation of total phenols in ricebean extracts, seed coat contained the highest amount of total phenolics i.e. 103.62 mg GAEg⁻¹. Rest of the extracts contained significantly reduced amount. Raw dal extract contained 45.10 mg GAEg⁻¹, cooked dal extract contained 21.95 mg GAEg⁻¹ and whole seed contained 52.15 mg GAEg⁻¹ phenolic content. For 100g sample, total phenolics in samples of the seed coat, raw dal, cooked dal and whole seed were 0.550 g, 0.271 g, 0.067 g, 0.480 g respectively.

hydrophilic phenols (ratio of approx. 1:1, w/w) respectively (Fig. 1). In the case of raw dal extract, it contained 12.87 mg GAEg⁻¹ hydrophobic and 32.23 mg GAEg⁻¹ hydrophilic phenols which constituted 29% (w/w) and 71% (w/w) of total phenolics (45.10 mg GAEg⁻¹). Similarly, cooked dal extract possessed 12.12 mg GAEg⁻¹ hydrophobic (55%, w/w) and 9.82 mg GAEg⁻¹ hydrophilic phenols (45%, w/w). Whole seed methanolic extract had total phenol with the value of 52.15 mg GAEg⁻¹ and it exhibited 60% and 40% (w/w) for hydrophilic (31.26 mg GAEg⁻¹) and hydrophobic (20.88 mg GAEg⁻¹) phenols respectively. Graphical representation for total hydrophobic and hydrophilic phenolic content (mg GAEg⁻¹) in ricebean extracts is given in Fig 1.

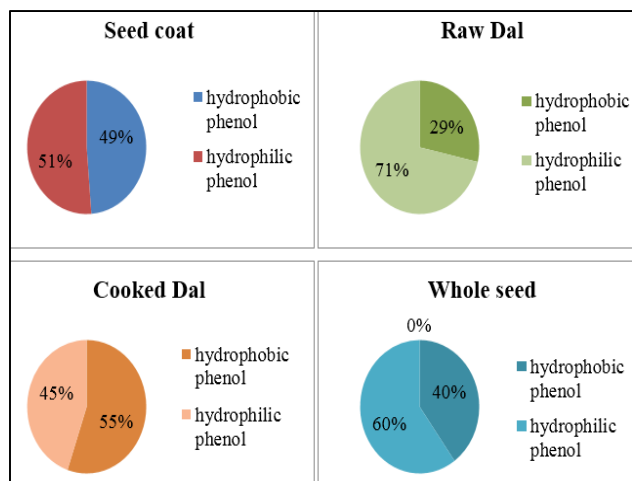


Fig 1: Total hydrophobic and hydrophilic phenolic content (mg GAEg⁻¹) in rice bean extracts

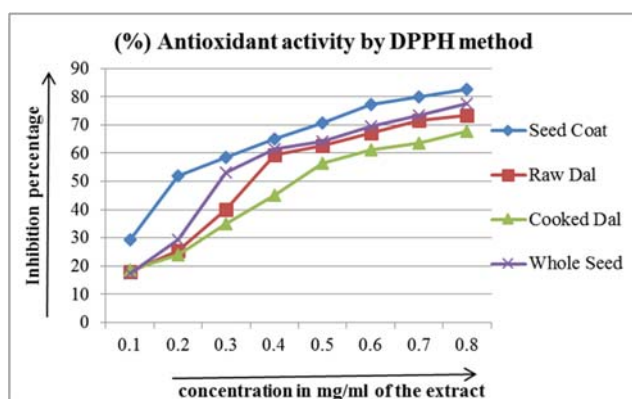


Fig 2: Inhibition % of rice bean extracts by DPPH free radical scavenging method

Estimation of flavonoid content in ricebean extracts

Here also, flavonoid content was highest in seed coat (25.32 mg CAEG⁻¹) analogous with the result obtained for total phenolics. Corresponding values for flavonoid content in raw dal, cooked dal, whole seed extracts were 33.66, 15.80, 30.19 mg CAEG⁻¹.

Evaluation of antioxidant activity in ricebean extracts

Evaluation of DPPH free radical scavenging activity

IC₅₀ values obtained by DPPH method in ricebean extracts were 0.17, 0.35, 0.43, 0.25 mg/ml for seed coat, raw dal, cooked dal, whole seed respectively. Statistically, IC₅₀ values for all the four extracts varied each other (Table 1). The corresponding maximum antioxidant activity (%) was 82.62, 73.34, 67.66, 77.60 as shown in Fig 2.

Evaluation of antioxidant activity by ferric thiocyanate (FTC) method

Similar to DPPH method's observations, here also IC₅₀ value of all the treatments of ricebean varied widely. The % of inhibition or antioxidant activity of methanolic extracts in theseed coat, raw dal, cooked dal, thewhole seed was 76.60, 65.80, 62.30 and 71.00 respectively. Corresponding IC₅₀ values for all extracts were 0.29, 0.65, 0.70, 0.57 mg/ml. Graphical representation of percentage inhibition value obtained by FTC method for all extracts of ricebean is shown in Fig 3.

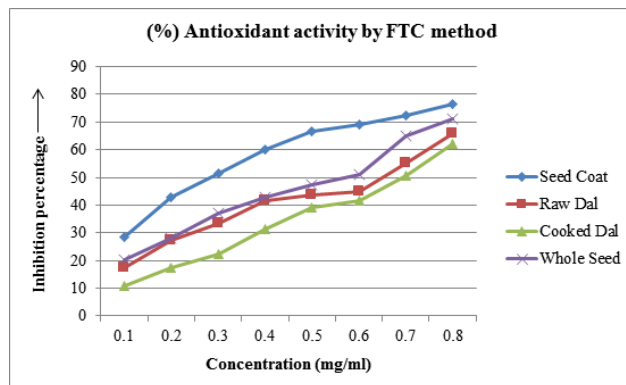


Fig 3: Inhibition % of rice bean extracts by ferric thiocyanate (FTC) method

Relationship between various phytochemicals and antioxidant activities of ricebean extracts

To know the implication of polyphenolic compounds in the antioxidant activities measured, the correlations were evaluated. The correlative values were obtained between DPPH free radical scavenging activity, antioxidant activity by ferric thiocyanate (FTC) method, in terms of IC₅₀ value and total phenols, o-dihydric phenols, hydrophilic and hydrophobic phenols and flavonoids in all the four extracts of the seed coat.

In seed coat extracts, total phenols were highly significantly correlated with hydrophobic phenols and IC₅₀ value by DPPH method with correlation coefficient value 0.990*, 0.966* respectively (where '*' shows significant at 5% level). A significant correlation (r = 0.961*) was analyzed between IC₅₀ values obtained by both the antioxidant activity methods. The corresponding correlation values obtained for raw dal extract showed that total phenols were highly significant with hydrophobic phenols, flavonoid, IC₅₀ value by DPPH method with correlation coefficient value 0.986*, 0.982*, 0.983* respectively. There was a high and positive correlation observed between IC₅₀ value by DPPH method and hydrophobic phenol (r = 0.988*). The correlation between flavonoid content and IC₅₀ value by FTC method was also positive and highly significant as r = 0.973*.

The correlation values determined for cooked dal extracts found that total phenols were highly significant with hydrophilic phenols and hydrophobic phenols with correlation coefficient value 0.985*, 0.989* respectively. Hydrophilic phenols and IC₅₀ value by FTC method were correlated with high positive and significant value (r = 0.952*). There was a high and positive correlation observed between IC₅₀ value by DPPH method and IC₅₀ value by FTC method (r = 0.980*).

The respective correlation values obtained for whole seed extract concluded that total phenols were highly significant with o-dihydric phenols and IC₅₀ value by FTC method with correlation coefficient value 0.974* and 0.958* respectively. Hydrophilic phenols were observed as significantly correlated with IC₅₀ value by FTC method (r = 0.978*). Rest of the correlation values obtained were found to be non-significant.

As per previous work is concerned, Yao *et al.*, (2012) [19] investigated the individual phenolic acids, the total phenolic content (TPC), the total flavonoid content (TFC), and the antioxidant and antidiabetic potential of 13 varieties of rice beans from China. Eight phenolic compounds (catechin, epicatechin, p-coumaric acid, ferulic acid, vitexin, isovitexin, sinapic acid, quercetin) were analyzed. In present studies, total phenolics including o-dihydric phenols, hydrophobic and hydrophilic phenols were observed to be decreased on

cooking. Segev *et al.*, 2010^[14], reported 85% decrease in total phenolics by cooking in *Kabuli* chickpea. Similarly, Hwang *et al.*, 2012, found that boiling and steaming significantly decreased the ascorbic acid content, total phenolics, and antioxidant levels compared with the other cooking methods. Reduced total phenolics in boiled or steamed foods have been attributed to the dissolution of phenolic compounds into the cooking water (Zhuang *et al.*, 2012)^[21]. Previous literature showed the phenolic composition of the seed coats and cotyledons of red lentils (Duen'as *et al.*, 2002)^[2] and the minimum and maximum levels for the different groups of compounds were presented. The seed coat contained high concentrations of dimer procyanidins, trimer procyanidins, hydroxybenzoic and hydroxycinnamic acids and trans-resveratrol glucoside which account for the higher amount of hydrophilic phenols as compared to hydrophobic phenols analogous for results found in rice bean. The variation in phenolic content is known to be due to genetic factors, the degree of maturity and environmental conditions. Secondly, extractability of different phenolic compounds is governed by the type of solvent (polarity) used degree of polymerization of phenolics, the interaction of phenolics with other food constituents, as well as the extraction time and temperature (Marathe *et al.*, 2011; Oomah *et al.*, 2011)^[9, 12]. Environmental factors such as growing season, soil type, temperature, seed variety and agronomic practices have also been found to significantly affect the phytochemical compositions (Tiwari *et al.*, 2011)^[17].

The relationship between polyphenolic compounds i.e. total phenolics, o-dihydric phenols, hydrophobic phenols, hydrophilic phenols, flavonoids and antioxidant activities is explained by finding the correlation between the polyphenolic compounds and activities. There was a positive and high correlation between total phenolics, flavonoids, and antioxidant activities. The relationship was highly significant in seed coat extracts. As explained earlier, the high concentrations of phenolic compounds, especially flavonoids, contained by the seed coat, are responsible for the greater antioxidant activity of this part of the seed compared to the cotyledon, with its much lower phenolic content. In order to elucidate this relationship in the lentil samples, the EC₅₀ values were correlated (Oomah *et al.*, 2011)^[12] with the relative percentages of quantified phenolic compounds, with respect to the total content of phenolics, in the seed coat and the cotyledon which was found to be highly significant. Also, the total phenolic content correlated positively with tartaric esters and flavanol contents indicating that extraction yielding high total phenolic content will result in extracts enriched in tartaric esters and flavanols. Furthermore, the mixture of phenolic substances in the extract may have synergistic effect, which is affected by varying test conditions. Some research workers who investigated different crops also reported that there might not be a significant correlation between flavonoids content and antioxidant activity (Pal *et al.*, 2011, Acharya *et al.*, 2012 and Meda *et al.*, 2013)^[13, 1, 10].

Conclusion

The selection of appropriate treatment for processing of food grains before consumption is a very important aspect to achieve the maximum concentration of desired phytoconstituents in suitable extracts. Dehulling and cooking are the important factors as the nature of phytoconstituents such as phenols and flavonoids present in pulses may be altered, which affects the antioxidant activity of pulse seeds. Therefore, the present study was undertaken to investigate the

effects of processing on the phenolic compounds and antioxidant activities of an underutilized pulse crop 'Ricebean'. In this context, it was concluded that phytochemical constituents and antioxidant activity were dependent on the processing method. Ricebean are found to be a better source of antioxidative phytochemicals. Therefore, suitably processed underutilized legume grains could be envisaged as a dietary ingredient in the formulation of supplementary foods with therapeutic values.

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