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Radical Scavenging Properties, Total Polyphenols and Flavonoids of Two Coloured Basidiomycetes from Four Pre-Determined Phases

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bstract
Prospects to evaluate appropriate harvest phase for edible basidiomycetes providing optimal value added health promoting benefits are limited in mushroom entrepreneurship. Two colored edible basidiomycetes (golden yellow *Pleurotus citrinopileatus singer* and pink *Pleurotus djamor R22*) were selected for the determination of Total Flavonoid, Total phenols, and free radical scavenging properties at spawn mycelia and three predetermined phases. Using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, antioxidative properties of young basidiocarps were relatively higher with EC_{50} values lower than mature fruiting bodies (3.176±0.905mg/mL against 10.787±1.007mg/mL) for *P. djamor* while *P. citrinopileatus* reported 7.321±1.291mg/mL against 10.502±1.017mg/mL. Antioxidant capacity collaborated the phenolics determined using Folin Ciocalteu Assay, young basidiocarps of *P. djamor* recorded higher values 12.655±1.019 against mature 4.317±0.921 mgGAE/ g dry weight) with *P. citrinopileatus* reporting 8.726±0.801 against 4.512±0.310 mgGAE/ g dwt. A strong correlation between phenols and the scavenging properties in aqueous extraction (R^2 =0.844, y=-0.700x +13.27 for yellow and R^2 =0.983, y=-0.896x +14.29 for pink oyster) was noted. Flavonoid contents did not show a linear correlation with the period, antioxidative, or phenolics properties. Early basidiocarps and basidiospores of basidiomycetes contain bioactive molecules worth targeting for value addition in formulating food supplements or pharmaceutical products.
Keywords: DPPH, growth phase, antioxidant, colored basidiomycetes, polyphenols, flavonoids, *Pleurotus djamor R22, P. citrinopileatus*

1.0 Introduction

Edible fungi are rich sources of health promoting molecules like polyphenols, flavonoids and radical scavenging properties (Ferreira *et al.,* 2009). The value added health benefits of edible basidiomycetes has been studied because they posses protein, minerals, vitamins, unique taste, and flavor (Pereira *et al.,* 2012; Reis *et al.,* 2012a; 2012b). In the past mushroom studies have attracted attention because of their ability to treat or prevent diseases (Chang, 1996). Studies shows that edible fungi in the genera *Pleurotus* spp, *Agaricus* spp, and *lentinula* spps exhibits antibacterial (Hirasawa *et al*., 1999; Oyetayo, 2009), antioxidative, anti-allergic, reduces blood cholesterol levels (Jeong *et al.,* 2010) anti-tumor (Ferreira *et al.,* 2010) and immunomodulatory effects (Valko *et al.,* 2007), and antioxidant properties (Mohamed, *et al.,* 2011; Soobrattee, *et al.,* 2005b). Besides, they have been documented as rich sources of bioactive molecules that confer health promoting benefits against degenerative heart diseases, cancers, and diabetes (Soobrattee, *et al.,* 2009; Cheung, *et al.,* 2003). These have elicited pharmacological and nutraceutical interests because inherent natural antioxidative and repair mechanisms that normally protect the body are inadequate to sufficiently prevent antioxidative stresses generated within (Simic, 1998; Barros *et al.,* 2008; Reis *et al.,* 2012b). Metabolisms in the body generate free radicals, which are chemically unstable and can damage cytoplasmic molecules like proteins, lipids, and DNA (Barros *et al.,* 2008). These radicals are manifested when the enzymatic systems protecting the body from oxidative stress is overwhelmed with excess generation of radicals like reactive oxygen species (ROS), which results in imbalances (Nijveldt, *et al.,* 2001). However, natural products with potent secondary metabolites would scavenge for such radicals (Dubost, *et al.,* 2007; Soobrattee, *et al.,* 2005; 2009). Health promoting benefits like antioxidant associated with polyphenols can add value to the product developed from potential sources like novel mushrooms (Scalber *et al*., 2005).

The main challenge is the possibility of producing mushroom products with maximum intact phenols, flavonoids and other bioactive molecules. Most entrepreneurs in this emerging industry have no idea on what stage the fruiting bodies should be collected to maximize on these benefits. Studies by Barros et al (2007a)

demonstrate that different parts of mushroom have differential health properties. However, little effort has been done to identify appropriate phase for their collection. It has been shown that mature fruiting bodies have reduced total number of bioactive molecules associated with radical scavenging properties (Wandati et al., 2013; Isabel, et al., 2004), but such attempts do not offer substantive directions. Isabel, et al., (2004) argue that as the fruiting body matures, bioactive molecules reduces because of their involvement in defense mechanism hence reduced extraction and bioavailability. Radical scavenging potential is largely attributed to phytochemicals like flavonoids and polyphenols that contribute largely to plant ecophysiology and survival against biotic and abiotic stressors (Soobrattee, et al., 2005). There are no evidence based suggestions or documented stages during which such molecules could be tapped, a gap this study attempts to address using pink and golden yellow edible basidiomycetes. Colored basidiomycetes like the Kenyan native golden yellow *Pleurotus citrinopileatus Singer* contains essential elements like minerals, amino acids, and vitamins (Musieba *et al.,* 2013) but phytochemical and antioxidative properties are yet to be reported, another gap we included in our study. Pinkish *P. djamor R22* is an exotic cultivar whose secondary metabolic profiles are yet to be explored as well.

2.0 Methodology

2.1 Sources of *Pleurotus spp*. Mushroom cultures

Two species of golden yellow *Pleurotus citrinopileatus* and *P. djamor* were used. *Pleurotus citrinopileatus* was acquired with the help of Dr. Fredrick Musieba of Kenya Industrial Research development Institute (KIRDI) while *P. djamor* was shipped from Plant Pathology department, Penn State University in Pennslyvania State, USA. These cultures were maintained and sub-cultured on Potato Dextrose Agar (PDA) medium and Malt Extract Agar (MEA). Spawns were developed using bird millet as the carrier media. Upon optimal colonization of the spawn (10 days) a spawning rate of 10% was used to inoculate 500g of both sugarcane baggase (*Saccharum officinarum*) and wheat straw (*Triticum aestivum*) substrates, which had been recommended as the best substrates (Wachira, 2003; Musieba, 2012). Pinning for both species took 14days; first early young fruiting bodies were harvested 2 days after opening the bags (16^{th} day after inoculation), the second batch of young fruiting body was collected a day thereafter (17^{th} day) and the third batch of mature fruiting bodies on the 20^{th} day. During these period coloration were noted to be intense hence the interest. A spawn mycelium was also included in this study

2.2 Sample preparation

Intact fruiting bodies of *Pleurotus citrinopileatus* and *P. djamor* mushrooms were dried in oven at 42^{o}C for 3days and milled to powder using electric miller. Ten (10g) of powder was placed in HPLC grade bottles and mixed with 100mL of distilled water and left in a 24 hour shaker set at 150rpm at room temperature in darkness. The liquid was decanted and the residue re-suspended in 100ml for re-extraction for 24hours. The filtrate was filtered through Whatman paper no. 1(12.5cm) and stored at 4^{o}C awaiting further analysis (Barros *et al*., 2008). The filtrate was reduced in volume (concentrated) using a vacuum evaporator (40^{o}C). These samples were analysed for total polyphenols, total flavonoids, and radical scavenging properties.

2.3 Total polyphenols (Folin-Ciocalteu Assay)

Calorimetric method according to Singleton and Rossi (1965), with a few modifications was used. Different concentrations of Gallic acid (Sigma) standard were prepared (0.1mg/mL to 1mg/mL). Briefly, aqueous extract (0.5mL) was transferred in a test tube and 2mL distilled water added. Folin-ciocalteu reagent, 1.25mL was added and after 5 minutes 6.25 mL of 20% aqueous sodium carbonate added to the mixture. This was vortex mixed and incubated for 40 minutes. One (1mL) of each preparation was diluted with 9mL of distilled water before reading absorbance at 725nm using UV-Vis-SDD-10AV SHIMADZU spectrophotometer against a blank containing all the reagents except the extract (instead used distilled water). Each experiment was done in triplicates. The total amount of phenolics was calculated as Gallic acid equivalent from the curve calibrated with Gallic acid as the standard. This was expressed as mg GAE/g dry weight of mushroom material.

2.4 Total flavonoids

Total flavonoid was determined using method described by Barros *et al.,* (2008) with a few modifications. Briefly, 1 mL of aqueous sample extract was aliquoted to the test tube, 4ml of distilled water was added, and after 3minutes 0.3mL of 5% sodium nitrite ($NaNO_2$) (Sigma) was added. The resulting solution was mixed allowed to stand at room temperature for 3 minutes. To this, 0.3mL of 10% $AlCl_3$ (analytical grade) was added. After 5 minutes, 2mL of 1M NaOH was added and shaken to react. This was diluted by topping up the solution to a volume of 10mL. A blank containing all the reagents except the extract was included in the set up (instead used distilled water as sample). Each experiment was done in triplicates. Absorbance was taken at 415 nm by UV-Vis-SDD-10AV SHIMADZU spectrophotometer. Quercetin (Sigma) standard was prepared in the range of 200ppm to 1000ppm, flavonoids content was expressed as mg Quercetin per gram dry weight (mg Qc/g DW).

2.5 DPPH Radical Scavenging activity

DPPH assay was based on Brand-Williams, *et al.,* (1995) with a few modifications. Briefly, 4mL of each extract concentration was used for this analysis, prepared as (1.5, 2.5, 5, 10, 20mg/mL) in deionised water, mixed with 1ml 0.1mM DPPH (Sigma) diluted in analytical grade ethanol. Fresh solution of DPPH was prepared in this ethanol. This was shaken in a test tube and incubated in the darkness for 45 minutes at room temperature. Ascorbic acid was used as positive control. The decrease in absorbance was measured at 517nm by UV-Vis-SDD-10AV SHIMADZU spectrophotometer. Each experiment was done in triplicates. Antiradical activity was expressed as percentage inhibition. The percentage inhibition of the radicals due to antioxidant properties of the extract was calculated as: (%) Inhibition = $[(A_{blank} - A_{sample}) \times 100]/A_{blank}$.

2.6 Statistical analysis

Results are represented as means triplicate ± standard deviation (means±SD). Linear regression was carried out to find out if there is any relationship between total phenols, total flavonoids, and radical scavenging activities in the extracts using version 16 of SPSS software. Based on Turkeys test, means at 5% ($P < 0.05$) level were considered significance.

3.0 Results and Discussions

3.1 Basidiocarps from different phases

Figure 1: showing fruiting body of predetermined harvest stage for both *P. djamor R22 and P. citrinopileatus singer*

Upon pinning, which took approximately 14days from inoculation, the first early young fruiting bodies were harvested 2 days after opening the bags (16^{th} day after substrate inoculation) as shown in figure 1a and 1b. The second set of samples labelled as young fruiting body was collected a day thereafter (17^{th} day after inoculation) as shown in figures 1c and 1d above. The last batch of mature fruiting bodies shown in figures 1e and 1f was collected on the 20^{th} day. These samples were used for phytochemical analysis.

3.2 Total Polyphenol content, Total Flavonoid content, and DPPH Radical Scavenging properties (RSA)

From table 1, the highest total phenol content (TPC) in *P. djamor* recorded during the Young Fruiting Body (YFB) stages was 12.655±1.019 mg GAE/ g dwt, while the highest total flavonoids content (TFC) was recorded during early young fruiting (EYFB) stage (9.612±1.306 mg QE/ g dwt). Spawn mycelia recorded higher TPC values than mature fruiting body (MFB); it is a considerable alterative for bioactive molecules. For *P. citrinopileatus* the highest TPC was 8.726±0.801 mg GAE/ g dwt determined during the YFB stages while the highest TFC was recorded during the same stage (6.471±1.205 mg QE/ g dwt). These findings shows EYFB and YFB are the better phases for obtaining both the basidiocarps and basidiospores than the MFB, which have reduced contents. These mushrooms also exhibited considerable Radical Scavenging activity (RSA); *P. djamor* shows a much lower value of 3.176±0.905mg/mL during the YFB than for *P. citrinopileatus* at 7.321±1.291mg/mL during EYFB phase. Our findings are within the reported ranges for various studies. For instance Wandati *et al.,* (2013) reports that Kenyan mushrooms have RSA values ranging from 0.58 to 4.58 mg/mL, while Barros *et al.,* (2007a) reported values ranging from 0.76 to 10mg/mL in Portuguese mushrooms. This shows that *P. djamor* has a better ability to scavenge for free radicals than *P. citrinopileatus* at both EYFB and YFB. However, other than maturity these properties depend on a number of other factors like parts of mushrooms, the species, and the substrates used for growing (Oboh & Shedohinde, 2009).

Early young and young fruiting bodies of colored basidiomycetes *P. djamor* and *P. citrinopileatus* are a rich source of TPC, TFC, and antioxidants than the Mature fruiting bodies ($p<0.05$). The TPC and RSA were significantly different between the young basidiocarps (EYFB, YFB) and the mature basidiocarps MFB for both species ($P \leq 0.05$). Although TFC and RSA did not show a proportional correlation, an increase in TPC is associated with increased TFC and reduced RSA. Other studies have also shown that high values of TPC are associated with low RSA (Wandati, *et al.,* 2013, Barros *et al.,* 2007a). Although TPC and TFC are associated with RSA, studies demonstrates that besides phenol compounds other molecules like carotenoids, tocopherols, and ascorbic acids, which have been reported in edible mushrooms before, may confer antioxidative properties (Barros *et al.,* 2007b). This study demonstrates that early young fruiting bodies (obtained 48 hours after pinning) and young fruiting bodies (24 hours thereafter) are rich source of TFC, TPC, and RSA for both *P. djamor* and *P. citrinopileatus*. This study collaborates previous finding by Isabel *et al.,* (2004) that reported variation of secondary metabolite between various harvest stages in button mushrooms. Our study used whole mushroom unlike Barros *et al*., (2007a), which majored on different parts of mushroom like caps and stipe. No study has reported TPC, TFC, and RSA of spawn mycelium exhibiting these properties.

This study shows that mycelium can be an alternative source of important bioactive molecules because mature fruiting bodies tend to have reduced values. Early collection of immature fruiting bodies is a major source of these compounds. The existing explanation for possible low values in the MFB is the possibility of phytomolecules being used in defensive processes against aging stressors (Isabel *et al.,* 2004). However, Sultana *et al.,* (2009) demonstrates that secondary metabolites vary depending on the solvent used for their extraction. Aqueous extracts may not give a complete profile hence our values could not be effectively compared with most studies that used methanol, ethyl acetate, and ethanol solvents.

Table 1: summary of TPC, TFC, and Radical Scavenging properties for water extracts of pink *Pleurotus djamor R22* and *Pleurotus citrinopileatus singer*

	Pleurotus djamor R22			*Pleurotus citrinopileatus singer*		
	Total Phenols (Folin – Ciocalteu assay) (mg/CE g of dry weight)	Total flavonoids (Aluminium Chloride colorimetric assay) mg QE/g dry weight	Radical Scavenging activity (DPPH assay)	Total Phenols (Folin – Ciocalteu assay) (mg/CE g of dry weight)	Total flavonoids (Aluminium Chloride colorimetric assay) mg QE/g dry weight	Radical Scavenging activity (DPPH assay)
Mycelia	7.708±0.047	7.403±1.001	6.769±1.007	6.516±0.221	4.387±0.606	8.051±0.065
1st period (EYFB)	10.908±1.052	9.612±1.306	4.547±0.43	8.112±0.017	6.039±0.489	7.321±1.291
2nd period (YFB)	12.655±1.019	8.014±0.703	3.176±0.905	8.726±0.801	6.471±1.205	7.710±0.404
3rd period (MFB)	4.317±0.921	8.840±0.088	10.787±0.652	4.512±0.310	4.921±1.003	10.502±1.017

Values expressed as mean±SD
EYFB- early young fruiting bodies
YFB- young fruiting bodies
MFB- mature fruiting bodies
TPC- Total polyphenolic content expressed as mg Catechin Equivalent (CE) per g dry weight of dry samples
TFC- Total flavonoids content expressed as mg Quercetin Equivalent (CE) per g dry weight of dry samples
RSA- Radical Scavenging Activity (DPPH), percentage inhibition converted to effective concentration able to scavenge 50% of the free radicals

3.3 DPPH Assay and the Correlation between TPC, TFC, and RSA

DPPH assay is a purple radical that reacts with hydrogen atoms in the sample to change color from purple to yellow (Brand-Williams, *et al.,* 1995). Sample extract acts as hydrogen donor, which makes it possible to screen for antioxidative activities in the mushroom extracts. Our results shown in Figures 1 and 2 shows that *P. citrinopileatus* and *P. djamor* are potential sources of hydrogen donating molecules scavenging for free radicals. These figures show that percentage inhibition increases with sample concentration (1.5, 2.5, 5.0, 10.0, 20.0 mg/mL) with the lowest effective concentrations lower than 20mg/mL (10.787±0.652 mg/mL for *P. djamor* and 10.502±1.017 mg/mL for *P. citrinopileatus*) as shown in table 1. These findings collaborates previous studies showing a similar trend in Kenyan mushrooms of 0.58 mg/mL to 4.58 mg/mL (Wandati *et al.,* 2013) and from other regions like Brazil 0.76 to 17mg/mL (Barros *et al.,* 2007a) and Mohamed et al., (2011). Ostensibly, the discrepancy in these various is attributed to the fact that most reported studies used non-polar solvent for their extraction, a fact that may affect the quantity of phytochemicals extracted (Sultana *et al.,* 2009).

From the linear correlation (illustrated in figures 3 and 4), a significant correlation (for *P. djamor* R^2 = 0.983, y= -0.896x+14.29 while for *P. citrinopileatus singer* R^2= 0.844, y= -0.700x+13.27) between TPC and RSA was noted across the harvest phase. However, no correlation was noted between Flavonoids and RSA, which conforms to other studies (Soobrattee, *et al.,* 2005; 2009). This demonstrates that phenolic compounds at early and young fruiting bodies have higher polyphenols than mature fruiting bodies. Mycelia from spawn could also be potential target for these value added properties. Barros *et al.,* (2007b) reported that phenolic compound and other secondary metabolites like carotenoids, tocopherols, and ascorbic acids determine RSA. However, if these metabolites are to be validated for additional functions, their targeted extraction with column chromatography, purification, determination of structure and alignment of data in database will shed more light for pharmacological application.

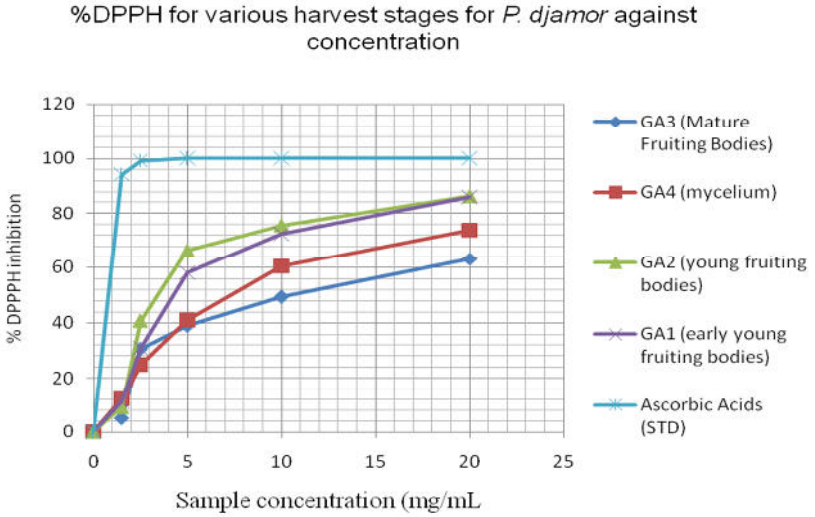

Figure 2: showing percentage DPPH inhibition for various harvest stages for P. djamor from 1.5mg/mL to 20mg/mL concentrations

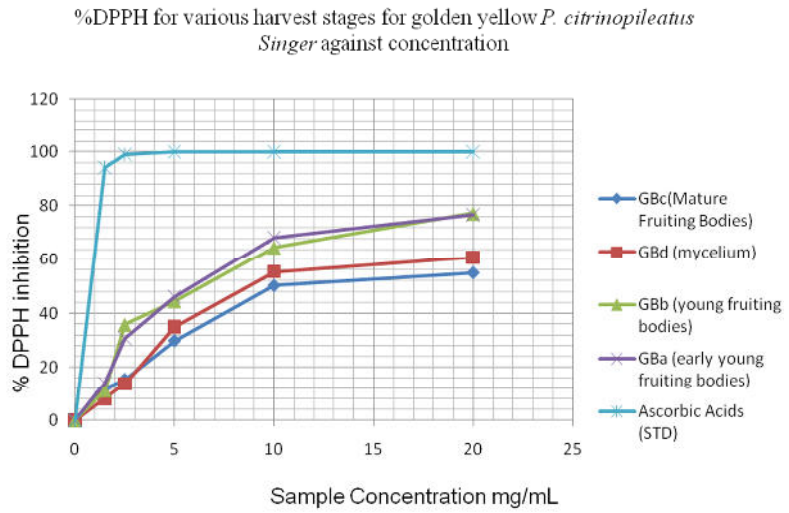

Figure 3: showing percentage DPPH inhibition for various harvest stages for *P. citrinopileatus* from 1.5mg/ml to 20mg/ml concentrations

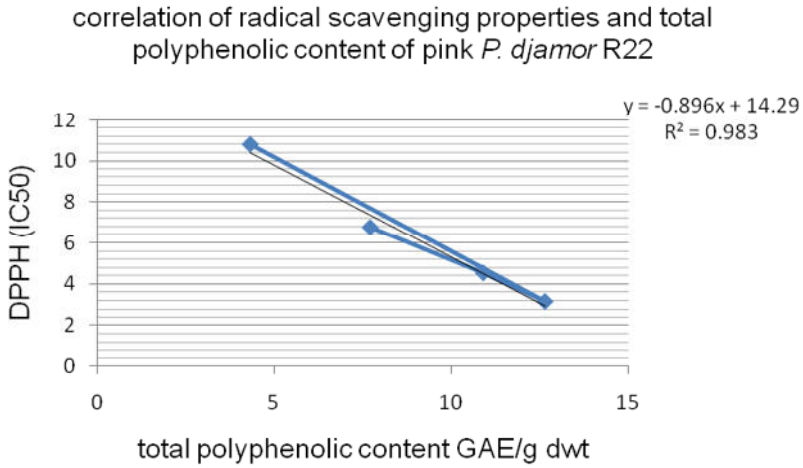

Figure 4: showing the correlation between Radical scavenging properties and Total polyphenolics contents of *Pleurotus djamor R22*

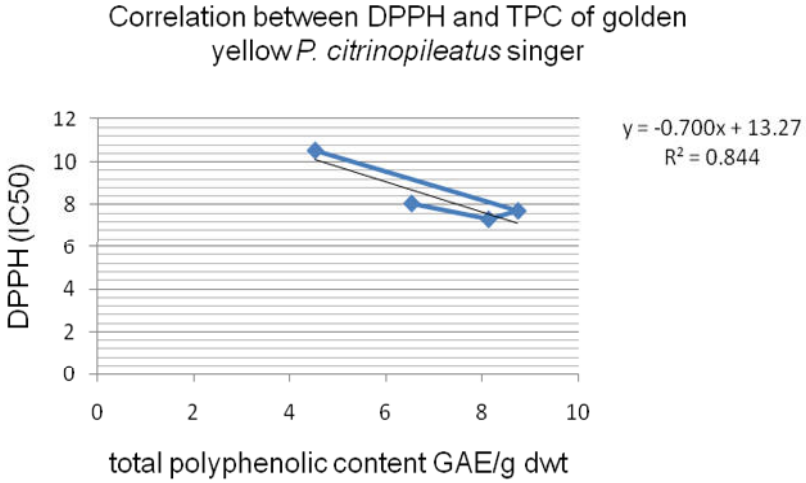

Figure 5: showing the correlation between Radical scavenging properties and Total polyphenolics contents of *Pleurotus citrinopileatus singer*

4.0 Conclusion

This study demonstrates that early young fruiting bodies, young fruiting bodies and the mycelium (immature

basidiocarps of pink and golden yellow oyster) contain immense secondary metabolites that can be targeted for development of pharmaceutical products and nutraceuticals. Other than collecting mushrooms during early growth stages, we recommend further isolation with non-polar solvents, characterization of these biomolecules, and determination of their structural profiles which may give additional information on their biological functions for *in silico* studies.

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