Research Article

Fraction of *Russula delica* Shows *In Vitro* Antioxidant Property Influenced by Polyphenols

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ABSTRACT

In many Asian countries, mushrooms have a long tradition of use as food due to high nutrition rich property. They have been also reported valuable as medicine in treatment of a wide variety of pathological damages which can be caused by oxygen-derived free radicals. The aim of this work was focused on obtaining phenol rich fraction from *Russula delica*, an edible mushroom, to get extract with high antioxidant potentiality. Result exhibited that the extract was able to scavenge reactive oxygen species even after boiling and extremely strong in searching hydroxyl radical. EC_{50} values ranged from 0.024 mg/ml to 2.26 mg/ml and was in the order of hydroxyl radical scavenging< chelating ability of ferrous ion< superoxide radical scavenging< $DPPH^{\cdot}$ scavenging< inhibition of β-carotene bleaching. Consequently, a preliminary chemical investigation was performed and revealed that the fraction was mainly composed of phenol. Ascorbic acid, β-carotene and lycopene were present in vestigial amount, whereas flavonoid was undetermined. Positive and strong correlation was also observed between antioxidant activity and total phenols (r= 0.988) implying that polyphenols was partly responsible for the antioxidant activity. Therefore these results indicated that the phenol rich extract of *R. delica* could be developed as a new health medicine for fighting against various human diseases.

Keywords: Antioxidant activity, correlation, edible mushroom, phenol, reactive oxygen species.

INTRODUCTION

Any atom or molecule possessing unpaired electrons in their outer orbit and derived from oxygen are defined as reactive oxygen species (ROS).[1] Several environmental factors such as pollution, pesticide, radiation and changes in lifestyle (tobacco smoke, extreme exercise) are responsible for ROS generation inside human body.[2] Although antioxidant defense and repair systems are available to fight against these radicals but overproduction of ROS can result in oxidative stress. These radicals can damage DNA, protein and membrane which can further contribute to several diseases.[3] Thus, consumption of dietary antioxidant will help to prevent free radical damage.[2]

In many Asian countries, mushrooms have a long tradition of use as food and medicine. They have been also described in treatment of a wide variety of pathological damages which can be caused by oxygen-derived free radicals.[4] Many studies have found that mushrooms can be used in the treatment of cancer[5], heart ailments[6], diabetes[7], gastric ulcer[8], hepatic damage[9], microbial pathogens[10, 11] etc. Several researchers have suggested that intake of polyphenol-rich foods and beverages may reduce risk of cardiovascular diseases, stroke and cancer as polyphenol possesses antioxidant properties.[12] Mushrooms are rich source of these secondary metabolites, so polyphenol rich extract from them may be used in treatment of these diseases.

Russula delica Fr., commonly known as milk-white brittlegill, is an edible ectomycorrhizal wild mushroom and distributed throughout the lateritic region of West Bengal.[13] The objective of this study was to prepare phenol rich extract from *R. delica* with potent antioxidant properties and determination of presence of different bioactive components.

MATERIALS AND METHODS

Mushroom sampling

Basidiocarps of *Russula delica* were collected in the month of July from lateritic region of West Bengal and identified using standard literature.[14] A voucher specimen (AMFH 600) has been deposited in the mycological herbarium of University of Calcutta, Kolkata, West Bengal, India.

Chemicals

All chemicals used were of analytical grade. L-methionine, nitro-blue tetrazolium (NBT), riboflavin, 2-Deoxy-D-ribose, ferric chloride, ferrous chloride, ferrozine, β-carotene, Tween 20, linoleic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate, Folin-ciocalteu reagent, aluminium nitrate, potassium acetate, acetone, n-hexane, standards such as L-ascorbic acid, ethylenediamine tetraacetic acid (EDTA), butylated hydroxyanisole (BHA), gallic acid, quercetin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Preparation of extract

Polyphenol rich fraction was extracted according to the method of Khatua *et al* (2013).[15] Dried and powdered basidiocarps of *R. delica* were extracted with ethanol at 25°C for 2 days to eliminate the alcohol soluble constituents such as coloured material, small organic molecules (steroid, terpenoids etc.) and fat. After filtration, the residue was then re-extracted with ethanol,

as described above. The filtrate was air dried, extracted by stirring with distilled water at 100°C for 8 hrs. After filtration, 4 volume of ethanol was added to the supernatant slowly and kept at 4°C overnight. Precipitate was discarded by centrifugation and the supernatant was concentrated under reduced pressure in a rotary evaporator. Concentrated polyphenol rich extract of *R. delica* (RudePre) was stored at 4°C until further analysis.

Superoxide radical scavenging assay

The scavenging potential of RudePre for superoxide radical was analyzed as described by Martinez *et al* (2001).[16] Each 3 ml reaction mixture sequentially contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (0.3, 0.5 and 0.7 mg/ml) of RudePre, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Reaction was started by illuminating sample with light and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in dark and served as blank. BHA was used as a positive control. The degree of scavenging was calculated by the following equation:

Scavenging effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 and A_1 were the absorbance of control and absorbance in the presence of sample respectively.

Hydroxyl radical scavenging assay

The method described by Halliwell *et al* (1987) was followed for this study.[17] The reaction mixture (1ml) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentrations (0.01, 0.03 and 0.05 mg/ml) of RudePre, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbic acid (100 µM) and H_2O_2 (1 mM). It was incubated at 37˚C for 1 h. 2ml TBA-TCA solution (0.375% (w/v) TBA, 15% (w/v) TCA and 0.25 N HCl) was added to stop reaction and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm against buffer. Identical tubes were kept where TBA-TCA solution was added prior incubation to subtract background colour. BHA was used as positive control. EC_{50} value expressed the effective concentration at which the scavenging free radical activity was 50%. Degree of scavenging was calculated by the following equation:

Scavenging effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 and A_1 were the absorbance of control and absorbance in presence of sample respectively.

DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of RudePre and a pure compound were measured from the bleaching of the purple coloured methanol solution of DPPH.[18] Various concentrations of RudePre (0.5, 1 and 1.5 mg/ml) were added to 2ml of 0.004% methanol solution of DPPH (w/v). After 30 min incubation period at room temperature in dark, the absorbance was read against a methanol blank at 517 nm. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. Degree of scavenging was calculated by the following equation:

Scavenging effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 and A_1 were the absorbance of control and absorbance in presence of sample respectively.

Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis *et al* (1994).[19] Different concentrations of RudePre (0.2, 0.4 and 0.7 mg/ml) were mixed with 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, absorbance of the mixture was determined at 562 nm against a blank. EDTA was used as positive control. EC_{50} value is the effective concentration at which ferrous ions were chelated by 50%. Percentage of inhibition of ferrozine- Fe^{2+} complex formation is given by this formula:

Chelating effect (%) = $\{(A_0-A_1)/ A_0\} \times 100$

Where A_0 and A_1 were the absorbance of control and absorbance in presence of sample respectively.

Inhibition of β-carotene bleaching assay

Antioxidant activity of RudePre was also evaluated by the β-carotene linoleate model system.[20] A solution of β-carotene was prepared by dissolving 0.5 mg β-carotene in 1ml HPLC grade chloroform. 25 µl linoleic acid and 200 mg Tween 40 were added to the solution. Chloroform was removed at 40°C under vacuum. 50 ml distilled water was added with vigorous shaking. Aliquots (2ml) of this emulsion were transferred into different tubes containing different concentrations of RudePre (1.5, 2 and 2.5 mg/ml) and absorbance was read at 490 nm to get zero time absorbance. The tubes were placed at 50°C for 2 h and again absorbance was taken. BHA was used as positive control. The antioxidant activity as percent inhibition rate of β-carotene bleaching relative to control at 2 h was calculated using the equation as follows:

Inhibition of bleaching effect (%) = $\{(D_0-D_1)/ D_0\} \times 100$

Where D_0 was the β carotene content after 2 h in case of control and D_1 was the β carotene content after 2 h in the presence of sample.

Total polyphenol contents determination

The content of total phenolic compounds in RudePre was estimated according to Singleton and Rossi (1965).[21] 1 ml of extract solution was mixed with 1 ml of Folin-ciocalteu reagent. After 3 min incubation, 1 ml of 35% sodium carbonate solution was added to the mixture and it was adjusted to 10 ml by water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm against blank. Gallic acid (10 – 40 µg) was used to calculate the standard curve. Estimation of phenolic compound was carried out in triplicate. The results were expressed as µg of gallic acid equivalents per mg of extract.

Total flavonoid estimation

Total flavonoid content was determined according to Park *et al* (1997).[22] 1 ml extract was diluted with 4.1 ml of 80% aqueous ethanol, 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1M potassium acetate. After 40 min incubation at room temperature the absorbance was measured at 415 nm. Quercetin (5 – 20 µg) was used to calculate the standard curve. Estimation of flavonoids was carried out in triplicate. The results were expressed as µg of quercetin equivalents per mg of extract.

β-carotene and lycopene estimation

β-carotene and lycopene were estimated according to the method of Nagata and Yamashita (1992).[23] 100 mg RudePre was mixed with 10 ml acetone-hexane mixture (4:6) for 1 min and filtered through Whatman no 4. Absorbance was measured at 453, 505, 663 nm. The assays were carried out in triplicate. Content of β-carotene and lycopene were calculated according to the following equations:

β-carotene (mg/100 ml): $0.216\ A_{663} – 0.304\ A_{505} + 0.452\ A_{453}$.

Lycopene (mg/100 ml): $-0.0458\ A_{663} + 0.372\ A_{505} – 0.0806\ A_{453}$.

Ascorbic acid determination

Ascorbic acid was determined by titration according to the method described by Rekha *et al* (2012)[24] with some modification. Standard ascorbic acid (0.1 mg/ml) was taken in a conical flask and made up to 10 ml by 0.6% oxalic acid. It was titrated against 2, 6-dichlorophenol indophenol dye which was prepared by adding 21 mg sodium bicarbonate and 26 mg dye in 100 ml water. The amount of dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid. Similarly, sample (concentration w µg/ml) was also titrated against dye (V_2 ml). Amount of ascorbic acid was calculated using the formula,

Ascorbic acid (µg /mg) = $[\{(10\ \mu g / V_1 ml) \times V_2\ ml\} \times w\ \mu g] \times 1000$.

Statistical analysis

Statistical analysis was done using Excel for Windows Software. A correlation coefficient, r, was established between phenolic contents and antioxidant properties of the extract using the same software.

RESULTS AND DISCUSSION

Superoxide radical scavenging activity

Among different ROS, superoxide anion is generated first.[25] Addition of one electron results in formation of this primary ROS. Harmful nature of superoxide lies in production of secondary ROS such as peroxynitrate (ONOO·), peroxyl radicle (LOO·), singlet oxygen, hydroxyl radical and hydrogen peroxide which are responsible for oxidative damage.[26] Thus, it is important to characterize scavenging ability of superoxide radical by antioxidants.

The chemistry behind method used by Martinez *et al* (2001)[16] is generation of superoxide radical from riboflavin in presence of light. The radical reduces yellow dye NBT to blue formazon. Thus the blue colour intensity is directly proportional to concentration of superoxide anion. In the present study, RudePre was found to be a notable scavenger of superoxide radicals (figure 1). EC_{50} value was determined as 0.675 ± 0.01 mg/ml which was much lower than phenol rich extract of *Russula laurocerasi* (RulaPre) (EC_{50} value= 1.56 mg/ml).[15] Polyphenol rich extract of *Inonotus obliquus* exhibited outstanding activity as EC_{50} value was less than 0.01 mg/ml.[27] RudePre showed significant correlation between phenolics and superoxide radical scavenging activity (r= 0.988) (table 1).

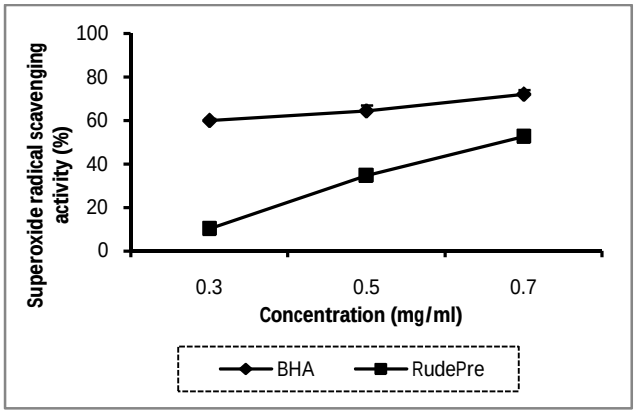

Figure 1: Superoxide radical scavenging activity of phenol rich extract of *Russula delica* (RudePre). Results are the mean ± SD of three separate experiments, each in triplicate.

Hydroxyl radical (OH^-) scavenging assay

Hydroxyl radical has a very short life and formed by an electron transfer from transition metals to H_2O_2. It is considered to be the most toxic among all ROS as the radical interacts with biomolecules immediately after formation. It can damage DNA by attacking purines, pyrimidines and deoxyribose.[3] Thus determination of scavenging ability of hydroxyl radical can be used for understanding antioxidant potentiality.

In our experiment, Fe^{2+}- ascorbate- EDTA- H_2O_2 system generates hydroxyl radicals (Fenton's reaction). Reaction between the radical and deoxyribose results in formation of malondialdehyde (MDA). MDA produces pink MDA-TBA chromogen which can be measured spectrophotometrically at 535 nm. When sample with antioxidant activity is added to reaction mixture, it removes hydroxyl radicals and prevents sugar degradation; as a result colour intensity decreases.[17] RudePre showed powerful hydroxyl radical scavenging activity which rose gradually with the increase of concentration (figure 2). The EC_{50} value was found to be at 0.024 ± 0.001 mg/ml. Phenol rich extract from another edible mushroom, *Tricholoma giganteum*, showed hydroxyl radical scavenging activity less effective than RudePre as evidence by its high EC_{50} value i.e. >0.8 mg/ml.[28] RudePre presented significant correlation between phenolics and hydroxyl radical scavenging activity (r= 0.99) (table 1).

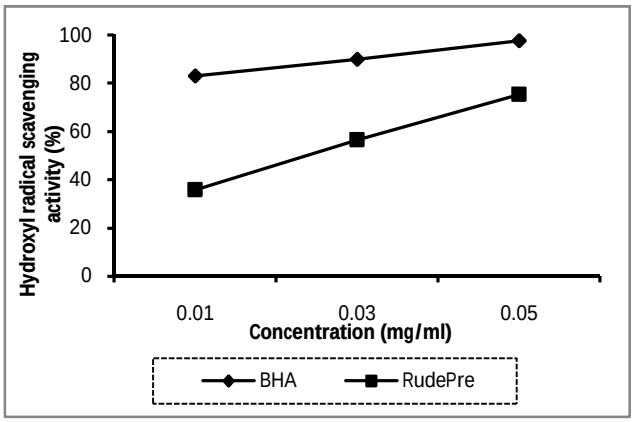

Figure 2: Hydroxyl radical scavenging activity of phenol rich fraction of *Russula delica* (RudePre). Results are the mean ± SD of three separate experiments, each in triplicate.

DPPH radical scavenging assay

DPPH radical scavenging is one of the most commonly used method to estimate antioxidant activity.[2] The advantage of this assay is that DPPH· is a commercially available radical which does not have to be generated before the assay, in contrast to many other scavenging assays.

DPPH· is a purple colored stable organic nitrogen radical, which after reduction changes to yellow colored diphenyl-picrylhydrazine. The radical shows maximum absorption at 517 nm. As antioxidant donates electrons to these radicals, the absorption decreases.[18] RudePre showed high DPPH radical scavenging activity (figure 3). EC_{50} of RudePre was at 0.87 ± 0.01 mg/ml whereas ascorbic acid presented EC_{50} value at 4.3 ± 0.3 µg/ml. RudePre showed better radical scavenging activity than methanol extract of *Russula nigricans* (EC_{50} 19.4 mg/ml) and *R. vinosa* (EC_{50} 21.26 mg/ml).[29] In the present test a significant correlation exists between total phenolics and DPPH scavenging activity (r= 0.996) (table 1).

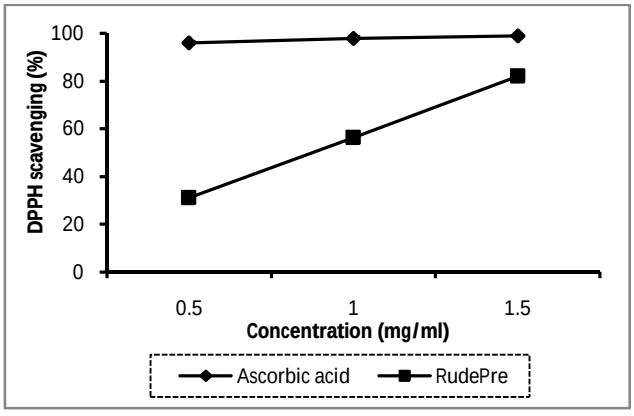

Figure 3: DPPH radical scavenging activity of phenol rich extract of *Russula delica* (RudePre). Results are the mean ± SD of three separate experiments, each in triplicate.

Chelating ability of ferrous ion

Chelating effect on ferrous ions is one of the important antioxidant activity mechanisms. Transition metals are known to generate hydroxyl radical by Fenton`s reaction. Thus chelating of ferrous ion may reduce iron-related free radical damage.[30] Chemistry behind this method is that ferrozine quantitatively forms complexes with Fe^{2+}; as a result colour of reaction mixture changes to purple. In presence of chelating agent, the complex formation is disrupted, thus resulting in reduction of intensity of colour. Reduction therefore allows estimation of the chelating ability of the existing chelator.[19] As shown in figure 4, the absorbance of Fe^{2+}-ferrozine complex was linearly decreased dose dependently. The percentage of metal chelating capacity at 0.2 mg/ml, 0.4 mg/ml and 0.7 mg/ml concentrations of RudePre were found as 19.23%, 36.39% and 51.55% respectively. However, EDTA showed about 95% activity at those concentrations. Methanol extract of pileus and stipe of *Russula griseocarnosa* showed EC_{50} at 2.33 and 5.99 mg/ml concentration respectively.[31] In the present study, significant correlation exists between total phenolics and ferrous ion chelating activity of RudePre (r = 0.978) (table 1).

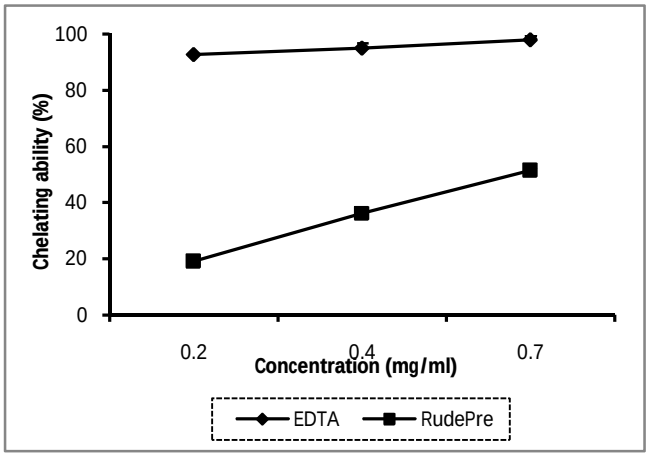

Figure 4: Ferrous ion chelating ability of phenol rich fraction of *Russula delica* (RudePre). Results are the mean ± SD of three separate experiments, each in triplicate.

Inhibition of β-carotene bleaching assay

This is one of the rapid methods used to screen antioxidants. Oxidation of β carotene and linoleic acid generates free radicals. Free radical formed upon the abstraction of a H_2 atom from one of its diallylic methylene groups attacks the highly unsaturated β carotene, hence β carotene is oxidized, losing its orange colour which is then monitored spectrophotometrically. Presence of antioxidant prevents β carotene oxidation and thus inhibits discoloration.[32]

A linear correlation was found between the concentration of RudePre and its antioxidant capacity (figure 5). At 1.5 mg/ml concentration RudePre exhibited 32.39% of inhibition of β-carotene bleaching which increased to 56.1% at 2.5 mg/ml level. EC_{50} value of RudePre was 2.26 ± 0.02 mg/ml whereas EC_{50} of phenol rich extract from *Russula laurocerasi* (RulaPre) was at 3.5 mg/ml.[15] RudePre shows high level of correlation between total phenolics and inhibition of β-carotene bleaching (r= 0.986) (table 1).

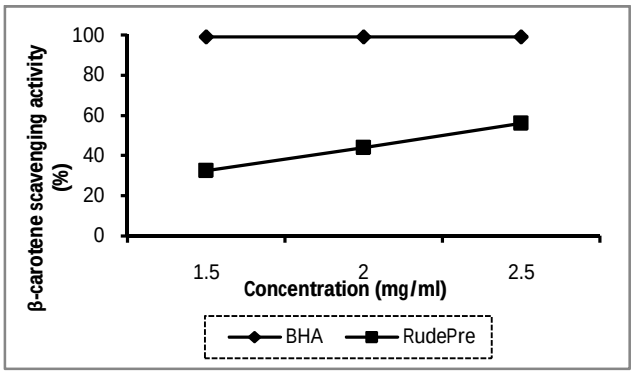

Figure 5: Inhibition of β-carotene bleaching activity of phenol rich fraction of *Russula delica* (RudePre). Results are the mean ± SD of three separate experiments, each in triplicate.

Table 1: Correlation coefficients for relationships between antioxidant activities and phenol

Assay	Phenol
Superoxide radical scavenging	0.988
Hydroxyl radical scavenging	0.99
DPPH scavenging	0.996
Chelating effect of ferrous ion	0.978
Inhibition of β-carotene bleaching	0.986
Mean	0.988

Antioxidant components

In the present study, table 2 shows total phenol, flavonoid, ascorbic acid, β-carotene and lycopene content in the extract of *R. delica*. Data displays phenol as the major antioxidant component whereas others are found in vestigial amounts except flavonoid. Flavonoid was undetermined in RudePre.

Table 2: Total phenol, flavonoid, ascorbic acid, β-carotene and lycopene contents of polyphenol rich extract of *Russula delica* (RudePre). Values are mean ± SD of three separate experiments each in triplicate. Total phenols are expressed in gallic acid equivalent (GAE), and flavonoids as quercetin equivalent (QAE). Nd= not detrmined

Phenol (µg /mg)	Flavonoid (µg /mg)	β-carotene (µg /mg)	Lycopene (µg /mg)	Ascorbic acid (µg /mg)
14.32 ± 0.56	Nd	0.48 ± 0.17	0.34 ± 0.11	2.49 ± 0.15

CONCLUSION

Results of the study indicated that phenol rich extract of *Russula delica* (RudePre) has powerful antioxidant property. Antioxidant effect of the extract was resistant to high temperature, even after boiling. RudePre showed excellent hydroxyl radical scavenging activity (EC_{50}= 0.024 mg/ml). While EC_{50} values of the extract for other assays ranged from 0.67 mg/ml for chelating ability of ferrous ion to 2.26 mg/ml for inhibition of β-carotene bleaching. RudePre contained mixture of bioactive components which is in the order of phenol< ascorbic acid< β-carotene< lycopene of which phenol showed strong association with antioxidant properties of the extract. Thus it can be suggested that RudePre may be used as a natural additive in food and pharmaceutical industries.

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